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Extracorporeal Removal of Plasma Hemoglobin in Sepsis

A Dissertation Presented

by

Jacob Ariel Levine

to

The Graduate School

In Partial Fulfillment of the

Requirements

For the Degree of

Doctor of Philosophy

in

Biomedical Engineering

Stony Brook University

December 2008

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Abstract of the Dissertation

Extracorporeal removal of plasma hemoglobin in sepsis

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Doctor of Philosophy

in

Biomedical Engineering

Stony Brook University

2008

Sepsis, a lethal syndrome, kills approximately 5.4 million people worldwide annually (Crit Care,2003.7(1):p.1-2). It is characterized by a systemic inflammatory response modulated by release into the circulation of cytokines and other toxins that cause organ damage. Hemoglobin is highly toxic when released by hemolysis into the plasma. Plasma hemoglobin levels become elevated during sepsis, and significantly increase cytokine production by macrophages (Shock,1998. 10(6):p.395-400; Shock,2002.17(6):p.485-90.) and mortality in animals with endotoxemia (Infect Immun, 1997.65(4):p.1258-66.). We built a bead (sorption) column to specifically remove hemoglobin by coupling the hemoglobin-binding protein, haptoglobin, to the beads. Breakthrough analysis data (that indicated favorable removal kinetics) was fitted to a Freundlich isotherm which was used to predict hemoglobin removal kinetics in a closed circuit. Accuracy of this prediction was verified ex vivo. The haptoglobin-column

efficiency (1-%toxin breakthrough) vs. column saturation was independent of flow (0.6-1 mL/min) and was not significantly altered when filtering hemoglobin from saline or haptoglobin-depleted plasma. These data indicate that hemoglobin removal kinetics can be predicted over a range of flows at physiologically relevant hemoglobin concentrations ex vivo, and suggest that column fouling should not be limiting in vivo. A hemoglobin solution (20mg/mL) was passed through either a haptoglobin or a sham-column, and the filtrates injected (0.3mL) into septic mice (cecal ligation and puncture). Fourteen day mortality was significantly lower in the filtered group (1/16 total) as compared to mortality in the sham group (10/17 total, $p < 0.01$). A miniaturized (4mL priming volume) extracorporeal circuit incorporating the column was developed to filter blood in a rat model of hemolysis. Rats were injected with hemoglobin (27.5mg/kg) and placed on extracorporeal circulation (1h at 1mL/min). The haptoglobin-column significantly reduced exogenous hemoglobin ($66 \pm 4\%$, $n=7$) as compared to the sham-column ($7 \pm 14\%$, $n=8$, $p < 0.01$), demonstrating the ability of the haptoglobin-column to remove hemoglobin in situ. Conclusion: We have thus developed and characterized a column that specifically targets hemoglobin and showed survival benefit of ex vivo filtration in a murine model of sepsis and demonstrated in situ elimination of hemoglobin from rats. This technology can be adapted to target one or multiple specific toxins in a number of diseases.

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List of Symbols

Q = flow
C = hemoglobin concentration
C₀ = initial hemoglobin concentration
C_{outlet}; C_{inlet} = concentration of hemoglobin at the column's outlet; inlet
V = volume of reservoir or intravascular space
G = generation rate of hemoglobin due to hemolysis
E = column efficiency at hemoglobin removal = $1 - C_{outlet}/C_{inlet}$
t = time
P = pressure
 ΔP = inlet pressure- outlet pressure
 μ = dynamic viscosity
 ρ = density
 ε = void fraction
 v' = superficial velocity
v = velocity
L = column height
D_S = spherical bead diameter
q = hemoglobin bound (mg)/ unit column (mg)
q_{max} = hemoglobin binding capacity (mg)/ unit column (mg)
 β = an empirically defined constant in Freundlich isotherm
 τ = shear stress
 γ = shear rate

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Acknowledgments

I would like to acknowledge the members of the Tracey lab who have supported this body of work in various ways, from teaching me techniques to providing helpful advice. I would specifically like to acknowledge Drs. Mahendar and Kanta Ochani for surgical support, Dr. Adam Rosebrock for assistance with the quantitative cytokine arrays, as well as NIH/NIGMS for financial support (to KJT). My committee members, Drs. Michael Hadjiargyrou, Richard Clark, and Edward Leonard, whose dedication to me, my body of work, and to the improvement of the human condition through basic discovery and translational research have been an inspiration to me during my training. I would like to express much gratitude to my principal scientific mentors: Yehuda Tamari, whose vision, hands-on approach, and attention to detail were invaluable in actualizing the project scope, the sorption column, and the miniaturized circuit; Dr. Kevin J. Tracey, whose wisdom, guidance, and optimism helped get me through both technical and emotional roadblocks. Finally, I'd like to thank my parents, wife, and children for their love, support, and sacrifice throughout my academic journey.

Chapter 1

Introduction

Numerous conditions exist in which an overabundance of biochemical species in the blood cause disease. In many cases, these species are produced in such large quantity that passive therapies cannot block pathological activity and no efficacious drugs have been developed. These pathologies include immunoglobulin-induced hyperviscosity syndromes such as Waldenstrom's macroglobulinemia and multiple myeloma, as well as protein deposition disorders such as cryoglobulinemia, and apolipoprotein (AA)-, immunoglobulin light chain (AL)-, and beta 2-microglobulin (dialysis related; DR)- amyloidosis, and acute disorders such as sepsis and septic shock.

To treat conditions such as these, a platform was developed for the specific removal of a single toxin or a combination of overabundant toxins through an extracorporeal blood-contacting device. Such a device would enable specific removal of large quantities of toxins, while retaining important beneficial and homeostatic blood elements. Additionally, it will avoid introducing drugs or large quantities of antibodies that might have their own toxicities.

As a practical application of this technology, the platform was tailored to remove plasma hemoglobin during sepsis. Sepsis is a syndrome in which patients may particularly benefit from this type of extracorporeal treatment. Whereas many of the above-listed diseases are chronic, sepsis is an acute disorder. Toxin removal will not permanently reverse chronic diseases; it will only provide symptomatic relief until the toxins accumulate again. In contrast, in acute diseases such as sepsis, the removal of significant toxin within a critical window may reverse established sepsis and help drive the individual back to a healthy state.

Background:

Overview of Sepsis

Despite the considerable effort of both basic science and clinical research programs, the treatment of sepsis in the critically ill, post-operative, and post-trauma patient populations remains a daunting problem [1], is the most common

cause of death in hospital intensive care units [2], is the most common cause of death in hospital intensive care units [3].

Sepsis syndrome is defined as systemic inflammatory response syndrome concurrent with proven or suspected infection. Sepsis is deemed “severe” when the associated immune system dysregulation leads to organ dysfunction. The initiating insult may be infection, traumatic injury, or perfusion reperfusion injury, but need not necessarily include bacterial elements [4]. When the cause is bacterial, the host’s inflammatory reaction is activated by motifs on bacterial surfaces, termed pathogen-associated molecular patterns. In Gram-negative bacteremia, lipopolysaccharide (LPS, endotoxin) with its lipid A moiety, is the main motif recognized by the innate immune system. Peptidoglycan, lipoteichoic acid, and toxic shock syndrome toxin-1 are the major activators of the proinflammatory cascades for Gram-positive bacteria, which contain no endotoxin [5]. These insults elicit a proinflammatory cytokine response that normally acts to contain the damage and instigate remodeling. Compensatory anti-inflammatory mediators are released, acting to restore homeostasis. However, if the compensatory measures are insufficient due to severity of the insult, systemic cytokine production can lead to a massive systemic inflammation through numerous amplifying cascade pathways. These can lead to hypotension, endothelial dysfunction (including increased microvascular permeability and loss of vasodilatory control), and activation of the coagulation system. These sequelae often lead to multiple organ dysfunction and failure [4].

Various treatments currently compose the standard of care for sepsis. Early, goal-directed therapy is applied, where continuous monitoring of venous oxygen saturation and blood pressure allows for tuning of these parameters by administering oxygen, mechanical ventilation, crystalloids, vasopressors, fluids, and transfusions to maintain predetermined levels. Mechanical ventilation may be provided at low tidal volumes to improve oxygenation while protecting the lungs from injury. Broad-spectrum antibiotics are generally administered before positive cultures are obtained, and narrowed when the pathogen is identified. Activated protein C, an anti-inflammatory, antiapoptotic, and anticoagulatory

drug, has also recently introduced as a therapeutic in sepsis. Additional supportive therapies often applied during sepsis include insulin therapy to control hyperglycemia and renal dialysis to compensate during acute renal failure [6]. There remains, however, much room for improved therapies to reduce mortality.

Systemic inflammation as a therapeutic target in sepsis

Twenty two years ago Tracey, et al. described the systemic inflammatory role of TNF in non-malignant disease [7], and published the first report using monoclonal anti-TNF antibodies as a therapeutic agent [8]. TNF was validated as a therapeutic target for septic shock by demonstrating that: 1) TNF is produced during septic shock [8]; 2) administration of TNF to normal mammals (including man) reproduces the hemodynamic, metabolic, immunological, and pathological sequelae of septic shock syndrome [7]; and 3) removing TNF from animals with septic shock syndrome by either pharmacological means or by the use of genetic “knock-out” technology prevents the development of lethal septic shock [9]. The same proof was never obtained for sepsis syndrome, however, because anti-TNF antibodies given during experimental Gram-negative peritonitis failed to improve survival. The failure of clinical trials targeting TNF may be due to an incorrect target for sepsis therapy or incorrect timing of the therapy. A different molecular target with a broader therapeutic window may be a more appropriate target for sepsis treatment [1, 10, 11]. Since production of TNF and other immunoregulatory molecules can be traced to elevated levels of extracellular hemoglobin [10, 12], it is highly probable that reducing extracellular hemoglobin levels will provide upstream protection against overwhelming inflammation.

Hemoglobin

Hemoglobin is the major oxygen transporter in the blood, making up 30% of the red blood cell. Hemoglobin A1, the vast majority of hemoglobin in the mammalian adult (~98%), is a protein consisting of two α chains (142 AA, 15.3 kDa) and two β chains (147 AA, 16 kDa)[13]. Each chain contains a single iron-centered heme group, essential for its interaction with O_2 , as well as CO_2 and H^+ .

Hemoglobin is efficient as an oxygen carrier due to its allosteric quality, binding and releasing oxygen cooperatively [14]. During sepsis, red blood cells become rigid [15, 16] and susceptible to hemolysis, generating an increased concentration of extracellular hemoglobin [17]. Elevated extracellular hemoglobin levels in septic rats has recently been observed in our lab as well, where levels are significantly increased over baseline by 24h post surgical peritonitis [Chapter 4].

Physiologic removal of extracellular hemoglobin

When red blood cells are destroyed within the intravascular space, hemoglobin enters the plasma. The hemoglobin rapidly dimerizes within the plasma becoming available to bind the liver-produced glycoprotein haptoglobin. Most physiologic extracellular hemoglobin clearance is mediated by haptoglobin, a tetrameric plasma protein of various phenotypic subunit associations that binds quickly and irreversibly (rate constant $\sim 5.5 \times 10^6 \text{ M}^{-1} \text{ Sec}^{-1}$, binding affinity $K_d \sim 1 \text{ pmol/L}$ [18]) to extracellular hemoglobin with 1:1 stoichiometry [19].

Haptoglobin is produced as an acute phase plasma protein by hepatocytes. It is synthesized as a single chain, which is then cleaved into two: an amino-terminal α -chain and a carboxy-terminal β -chain of 40 kDa. There is a human haptoglobin variant within the α -chain ($\alpha_1, 8.9 \text{ kDa}$; $\alpha_2, 16 \text{ kDa}$) [20], leading to three phenotypically different polymorphisms. These include the homozygous 1-1 which forms only linear dimers, 2-2 which forms cyclic multimers, and the heterozygous 2-1 which forms linear dimers as well as multimers. The efficiency of the antioxidative properties of haptoglobin may be dependent on the predominance of haptoglobin type, perhaps due to the size and shape of each variant [21-23]. A neo-epitope is formed through the binding of haptoglobin to extracellular hemoglobin that is recognized by CD163, a transmembrane scavenger receptor [24] found almost exclusively in tissue macrophages residing mostly in the liver, spleen, and lymphoid tissue [25]. The haptoglobin levels in normal human plasma range from 27-139 mg/dL [26]. As haptoglobin is not

regenerated after delivering hemoglobin for degradation, extracellular hemoglobin levels quickly rise when the circulating haptoglobin is overwhelmed.

Complexation with haptoglobin prevents the uptake of hemoglobin in the kidneys, as the size of the complex is too large for filtration. If the haptoglobin system is overwhelmed, extracellular hemoglobin enters the kidneys, causing harmful iron loading and peroxidative tissue injury [18]. The hemoglobin may either be excreted in the urine or reabsorbed by the proximal tubular cells through endocytic receptors (megalin, cubilin). The hemoglobin is degraded and the iron stored through intracellular ferritin binding [27, 28].

Ferric heme may spontaneously dissociate from globin in the plasma. Heme is physiologically catabolized by multiple pathways: through heme oxygenases (HO) and through extra-HO pathways. Heme-oxygenase is a 32 kDa enzyme which converts heme to biliverdin, CO, and Fe^{2+} [29, 30]. Biliverdin is further converted to bilirubin by biliverdin reductase, while the free iron is absorbed and recycled by ferritin. HO and the oxygenation byproducts have all been shown to be cytoprotective against oxidative injury [29].

The extra-HO pathways include removing heme through protein sequestration and through degradation of the heme. Hemopexin is the predominant heme-binding protein in the plasma. It is a 60 kDa glycoprotein which binds to heme ($K_d < 1 \text{ pM}$; [31]) in an equimolar ratio, forming a complex which reduces heme toxicity. The hemopexin shuttles the heme into the liver via CD91 receptors [32], where it is degraded [33]. Circulating albumin, high and low density lipoproteins, and intracellular heme-binding protein are other proteins that complex with heme, reducing toxicity [27]. Proteins that have been shown to directly degrade heme include alpha 1-microglobulin and reduced glutathione [30].

Plasma hemoglobin as a Therapeutic Target during Sepsis:

Extracellular Hemoglobin as a Modulator of Oxidative, Apoptotic, and Hematologic Damage

Plasma hemoglobin is now recognized to be a biologically dangerous compound, largely due to its iron-mediated oxidizing capabilities [34-36]. Hemoglobin, normally encapsulated within erythrocytes, is suspended in a concentrated milieu of antioxidants, such as ascorbate, superoxide dismutase, catalase, glutathione peroxidase, methemoglobin reductase, and reducing molecules produced by glucose-6-phosphate dehydrogenase [35]. Outside the erythrocyte, the reducing environment is far more dilute. Rampant oxidation to methemoglobin [37] (heme iron Fe^{2+} oxidized to Fe^{3+}) and continuous formation of powerfully oxidizing hydroxyl radicals then takes place through a cyclic Fenton/Haber-Weiss reaction [34]. Production of hydroxyl radicals and the enhanced peroxidation of low-density lipoproteins and polyunsaturated fatty acids (such as arachidonic acid), can lead to changes in the cell membrane that initiate intracellular signaling cascades. This signaling causes movement of nuclear transcription factors leading to altered regulation of redox-sensitive genes, to release of cytokines such as TNF, or to cell death [34-36]. In conditions that can lead to sepsis, such as endotoxemia or bacteremia, the rate of oxidation of heme iron is increased [38].

In addition to the oxidative damage caused by the heme iron-containing extracellular hemoglobin, vascular damage has also been attributed to heme-depleted globin. Tsemakhovich, et al. have demonstrated endothelial cell damage due to globin by culturing endothelial cells in 20 μM globin. The authors suggest that the vascular damage is likely a result of the strong hydrophobic pocket revealed by heme displacement from globin. The hydrophobicity of the globin results in adsorption to endothelial cell membranes followed by membrane penetration through adsorptive pinocytosis, which can distort the cell and even lead to its rupture [36].

In addition to oxidative and apoptotic damage, extracellular hemoglobin can cause blockage of small blood vessels leading to ischemia and organ damage. Much of this damage is attributed to the potent nitric oxide scavenging of extracellular hemoglobin. Nitric oxide is an important vasoactive molecule that prevents blockages in the microvasculature. Oxygenated hemoglobin quickly

converts nitric oxide to inactive nitrate (rate constant = $3.4 \times 10^7 \text{M}^{-1} \text{sec}^{-1}$ [39]) and deoxygenated hemoglobin binds nitric oxide to form heme-nitrosyl-hemoglobin [40]. Normally, nitric oxide is protected from rapid inactivation by the diffusional resistances inherent in the sequestration of hemoglobin within the red blood cells as well as the cell-free boundary layer (1-2 μm) adjacent to the endothelium (in vessels with diameters $>20\mu\text{m}$) [41]. These resistances disappear once hemoglobin emerges from the cell. Nitric oxide, when normally produced by endothelial cells in response to stimuli such as hypoxia, enables guanylyl cyclase in neighboring vascular smooth muscle cells to produce cyclic GMP allowing smooth muscle cell relaxation and vasodilation. The nitric oxide scavenging of extracellular hemoglobin prevents this cGMP production and thus prevents smooth muscle cell relaxation and vasodilation [42]. The simultaneous decrease in cGMP production in platelets leads to increased platelet activation and aggregation [29] which is particularly thrombogenic in a region of local vasoconstriction. Additionally, extracellular hemoglobin can cause blockages of small vessels by upregulating the endothelial conversion of arachidonic acid to 8-iso prostaglandin $\text{F}_2\alpha$ [43], and increasing production and secretion of endothelin-1 [44], both potent vasoconstrictors. Simultaneously, methemoglobin induces the production of IL-6, IL-8 and E-selectin by endothelial cells [45] perhaps contributing to increased activation and recruitment of neutrophils during hemolysis [46]. Heme, which is released by methemoglobin, has also been demonstrated to activate neutrophils [47] as well as increase vascular permeability, adhesion molecule expression, and granulocyte invasion [48]. Cell-free hemoglobin developed as an oxygen-transporting resuscitation fluid has resulted in toxicities such as hypertension, bradycardia, decreased kidney function, fever, intravascular coagulation, and ischemic parenchymal damage [49]. Elevated extracellular hemoglobin has been implicated in kidney tubular epithelial cell injury, hepatic necrosis, neuronal cytotoxicity, activation of mononuclear leukocytes causing intravascular coagulation and cytokine release, coronary and pulmonary restriction, and increased mortality in conjunction with endotoxemia [50].

Elevated plasma hemoglobin may also contribute to the proliferation of pathogens in the blood, as many organisms, such as *Haemophilus influenzae* [51] and *Vibrio vulnificus* [52], use or rely on the iron from hemoglobin to thrive. The immunological response to bacteremia and severity of developed sepsis increases relative to the bacterial load.

Extracellular Hemoglobin as a Modulator of Inflammatory Damage

Extracellular hemoglobin has also been implicated in the development of inflammatory disorders. Conditions that are associated with increased extracellular hemoglobin (cardiopulmonary bypass, burn trauma, stored red cell transfusion) are often complicated by the development of systemic inflammation [10]. Chronic renal dialysis patients, whose cardiovascular comorbidities are mainly attributed to inflammation [53, 54], are at elevated risk for hemolysis as their blood is routinely subjected to mechanical forces while their uremic state reduces erythrocyte deformability [55]. Also, the lethality of gram-negative bacteremia is increased by hemolysis [12].

Plasma hemoglobin is particularly dangerous in a clinical state of sepsis, as hemoglobin has been demonstrated in our lab and in others to act synergistically with bacterial endotoxin lipopolysaccharide (LPS) resulting in increased toxicity and systemic inflammatory response. Our lab has shown through *in vitro* studies that coadministration of hemoglobin in concentrations ranging from 1 to 1000 $\mu\text{g}/\text{mL}$ and LPS induce a multifold increase of cytokines (TNF, IL-1 α , IL-1 β , IL-2, IL-6, IL-10) release by macrophages over that induced by LPS alone [10, 56]. Roth and colleagues have shown extracellular hemoglobin-enhanced LPS activation of coagulation through upregulated production of tissue factor by peripheral blood mononuclear and endothelial cells [57, 58], as well as by binding of LPS to endothelial cells [59]. They demonstrated that when endothelial cells in culture were challenged with LPS (0.1 $\mu\text{g}/\text{mL}$) and hemoglobin (10 mg/mL) there was a five-fold increase in tissue factor production compared to challenge with LPS alone [59]. When peripheral blood mononuclear cells were cultured with LPS (0.1 $\mu\text{g}/\text{mL}$) and hemoglobin (60 mg/mL), there was a 7.3-fold

increase in tissue factor “units” (95 vs. 13) over those cultured without hemoglobin [58]. The authors conclude that extracellular hemoglobin acts synergistically with LPS to stimulate procoagulant activity.

In vivo, it has been observed that the administration of cell-free hemoglobin significantly increases mortality in murine polymicrobial sepsis. Our lab has recently shown mortality increase dose-dependently as mice with perforated ceca are injected intravenously with increasing amounts of hemoglobin (20-2000 mg/kg hemoglobin; 30-100% mortality in hemoglobin-administered animals vs. 0% lethality in saline-administered controls) [Chapter 4]. Others have demonstrated extracellular hemoglobin significantly decreases survival in models of endotoxemia. Su, et al. injected endotoxemic mice with 1.8 g/dL hemoglobin intravenously at 10 hours after intraperitoneal administration of LPS, and found that overall survival was diminished (20% lethality in controls vs. 100% lethality in hemoglobin-administered animals). The authors concluded that the administration of cell-free hemoglobin to patients with endotoxin exposure was dangerous [60]. Additionally, White, et al. have demonstrated that coadministration of hemoglobin (1.75 g/kg \approx 4 g/dL) caused 100% lethality in a rabbit model using a sublethal dose of LPS (14.5 μ g/kg) that only caused some hematologic changes (such as thrombocytopenia) in the control group. Moreover, when the dose of LPS was 1,000-fold less (14.5 ng/kg), coadministration of hemoglobin caused 50% lethality whereas no toxicity was detected in the control group [61]. They concluded that the combination of intravenous endotoxin and hemoglobin can be catastrophic and that the administration of extracellular hemoglobin to trauma victims who are susceptible to infection is dangerous. Though some studies above utilized much higher concentrations of hemoglobin than expected in septic patients, the drastic effect on clinical outcome is suggestive of the negative impact extracellular hemoglobin can have in the sick.

These assessments are supported by the work of Hébert, et al., whose large controlled prospective studies on the efficacy of blood transfusions in critically ill patients have shown that the mortality was higher among patients who

received more transfusions (transfusion trigger of 10 g/dL hemoglobin) than those who received fewer transfusions (transfusion trigger of 7 g/dL hemoglobin) [62], and that the severity of multiple organ dysfunction increased with increasing transfusions ($P=0.02$) [63]. These results may be due to the extracellular hemoglobin that accumulates over time within stored red blood cell units [64, 65]. Additionally, extracellular hemoglobin is thought to be a contributing factor in the development of acute respiratory distress syndrome, increasing mortality in septic patients [46].

The ability of hemoglobin to synergize with LPS may be due to hemoglobin binding bacterial LPS. It has been demonstrated that hemoglobin forms stable complexes with many types of LPSs, causing disaggregation of LPS multimers [49, 56, 57]. The bioactivity of the LPS may therefore be enhanced by exposing more of the LPS lipid A moiety to the environment. Additionally, Fourier-transform infrared-spectroscopic experiments have indicated that when LPS binds hemoglobin, the lipid A acyl chains alter inclination, which changes the shape, and possibly alters the reactivity of the active lipid A moiety [66]. Looking from an alternate perspective, the synergistic effect may be due to the oxidizing activities of LPS on hemoglobin [67], resulting in enhanced toxicity on the endothelium by oxidants created by the abovementioned hemoglobin iron [68].

Taken together, extracellular hemoglobin, which can be elevated during sepsis, significantly increases oxidative damage, levels of circulating cytokines, and lethality during endotoxemia. We hypothesize that decreasing the level of extracellular hemoglobin will mitigate hemoglobin's toxic effects during sepsis, decreasing tissue damage, cytokine levels, and mortality.

Extracorporeal removal of extracellular hemoglobin

The specific removal of extracellular hemoglobin from the septic patient has not previously been considered. Plasma hemoglobin's damaging oxidative effects and powerful stimulation of systemic inflammation and lethality in the presence of gram negative bacterial LPS make it quite dangerous for patients with polymicrobial sepsis. However, it is often present in the circulation of septic

patients in quantities unmanageable by passive immunization therapies. Injectable haptoglobin would also not likely be efficacious as circulating haptoglobin-hemoglobin-LPS complexes may still be dangerously bioactive. In Japan, where injectable haptoglobin has been used clinically to treat hemolysis, there have been conflicting reports on its efficacy at reducing extracellular hemoglobin, with some groups claiming that extracellular hemoglobin levels dropped rapidly upon administration of haptoglobin, while others observed no significant change in extracellular hemoglobin levels or extent of renal damage [69-71]. In addition, reducing extracellular hemoglobin by injecting haptoglobin would require a large dose, on the order of 1-10 grams. This quantity is likely to cause toxicity, immunological irregularities, and liver damage. Lastly, the regulatory hurdles to develop a protein as large as haptoglobin as an injectable are significant. By contrast, the removal of extracellular hemoglobin via extracorporeal filtration should be much easier and less costly.

Extracorporeal devices for sepsis

Extracorporeal devices are widely used to treat renal disease patients and support patients undergoing cardiac surgery. There have been numerous studies in which extracorporeal devices have been used as a therapy for sepsis as well. These include continuous hemodialysis, hemofiltration, hemodiafiltration, plasmapheresis, and hemoperfusion [72]. This review will focus on hemoperfusion as a therapy for sepsis and/or endotoxemia.

Hemoperfusion

Hemoperfusion or hemoabsorption describes an extracorporeal technique in which an adsorptive surface is placed in direct contact with blood or plasma. Hemoperfusion removes substances from blood, specifically or nonspecifically, but does not transfer fluid (other than the priming solution). In contrast to hemodialysis, hemofiltration, hemodiafiltration, and plasmapheresis, hemoperfusion is able to remove toxins specifically without introducing foreign material into the bloodstream. Additionally, hemoperfusion can be designed to remove only the toxins of interest, retaining crucial nutrients as well as beneficial

and homeostatic blood elements. In the absence of kidney failure, the removal of fluid is not warranted, hence hemoperfusion can be delivered more economically than the dialysate-intensive therapies. In the presence of acute renal failure, a hemoperfusion module can be post-fitted onto a standard hemodialysis/filtration device.

There has been much work done on hemoperfusion as a therapy for sepsis. These attempts can be divided into two main categories: broad and narrow spectrum hemoperfusion.

Broad spectrum hemoperfusion

There are three classes of broad spectrum hemoperfusion devices that have been used in the context of sepsis therapy. These are uncoated activated carbon, coated activated carbon, and polymer resins. Activated carbons have been used for hemoperfusion since the 1960s [73] due to their high adsorptive capacity and enormous surface area. They have been looked at in context of endotoxin removal in the 1970s and 1980s [74, 75]. However, carbon hemofilters in clinical settings were plagued by hemoincompatibility issues (thrombocytopenia and neutropenia). These spurred the development of coated carbon hemofilters, which isolated the blood formed elements from the activated carbon surface [76]. These coatings are less hemoincompatible, but significantly reduce the adsorptive capacity of the filter, especially with regard to middle-weight and larger molecules (>300 Da) [77, 78].

Researchers who used uncoated activated carbon focused on demonstrating capacity for cytokine removal, manipulating pore size, and elucidating bioincompatibility. Howell, Sandeman, et al. reported that pyrolysed carbon matrices reduced the cytokines TNF, IL-6 and IL-8 ex vivo [79]. That group also noted that although uncoated carbon increased leukocyte adhesion ex vivo, it was actually a passivating surface and did not activate the clotting cascade [80]. Yushin, Hoffman, et al. noted that the pore size of the material is controllable, allowing tailoring for increased selectivity for cytokines of certain sizes [81]. Malik, Warwick, et al. showed that by engineering mesoporous

structures (2-50 nm pore diameter) into uncoated carbon hemofilters, the carbon filters become more efficient at adsorbing middle molecules such as IL-1 β [77, 82].

Coated carbon perfusion devices have not been studied as thoroughly in sepsis, as the size of most circulating cytokines in sepsis make those devices inefficient. However, some have been evaluated for ability to reduce endotoxin, including charcoal coated with agarose and chitosan-PEG [83, 84]. Other coated carbon devices that have been developed include carbon coated with silicon rubber polyurethane, polyvinyl acetate [85], cellulose (Adsorba 300C) [79], polyhydroxyethyl methacrylic acid (Poly-HEMA, hemosorba, JP), and heparin hydrogel (Clark R&D) [78].

Polymer resins have been designed to have a large adsorptive surface like carbon and are more easily manipulated. Davankov, et al. reported that they engineered a hypercrosslinked styrenic polymer filter with mesoporic structures and observed a 65% reduction in B2-microglobulin with minimal loss of albumin from blood of patients with chronic kidney disease. This selectivity was due to size exclusion of the pores. That group has since increased the hemocompatibility of the polymer by coating the surface with hydrophilic groups [86]. Another example of a broad spectrum polymer hemoadsorber is CytoSorb™. Kellum, et al. performed hemoperfusion studies in endotoxemic rats using the CytoSorb™ cartridge containing 10 g of polystyrene divinyl benzene copolymer beads with a biocompatible polyvinylpyrrolidone coating. They noted an increase in survival time of 21% (629 \pm 114 min vs. 518 \pm 120 min) in lethal endotoxemia. That polymer did not reduce LPS from the blood, however it did reduce IL-6, IL-10, and TNF [87].

Narrow spectrum hemoperfusion

In contrast to broad spectrum hemoadsorbents, numerous hemoadsorbents have been designed to remove specific toxins from the blood. This class of adsorbents allows for the retention of nonspecific protein along with the removal of the toxin of interest.

For sepsis, which is often preceded by endotoxemia, interest in the specific removal of endotoxins has been high, and was reviewed [88] in 2001. Amoureux, et al. have shown that using the endotoxin-binding protein Ofloxacin on crosslinked agarose beads can remove endotoxin from plasma (breakthrough at about 8 bed volumes of plasma with 50% efficiency). There was minimal hemoincompatibility, with no activation of coagulation, absence of consumption of coagulation factors, no loss of fibrinogen, and no complement activation [89, 90]. Others have used beads coated with charged anionic ligands such as lysine [91, 92], polyethyleneimine or diethylaminoethyl which effectively removed endotoxin by electrostatic interaction [93, 94]. Albumin-coated (acrylic) beads were developed (Endotoxin Adsorber EN500, Fresenius) to selectively remove LPS. This technology had promising preclinical results, reducing the limulus amoebocyte lysate (LAL) activity of a sample from 8.59 ± 2.07 EU/ml (mean \pm SD) to 1.82 ± 0.77 EU/ml [95]. A small phase I study with SIRS patients demonstrated efficient LPS removal and increased Apache II score [96], but the phase II pilot (145 patients) demonstrated no significant benefit in survival or improvement in clinical scores [57].

Another major effort at removing endotoxin from septic blood has been with using polymyxin B (another endotoxin-binding protein) bound to polystyrene-based fibers packed in a cartridge (DHP-PMX™) [97]. This filter has undergone numerous preclinical as well as clinical tests as a hemoperfusion device in sepsis to mixed results. In a model of septic pig neonates, PMX increased systemic blood pressure, reduced IL-6, HMGB1, and LPS while slightly increasing survival time (12.2 ± 1.1 hour vs. 9 ± 0.7 h) [98]. It was shown that PMX was able to reduce reactive oxygen species (ROS) after PMX perfusion, perhaps by direct adsorption or secondary to LPS removal [99, 100]. In an uncontrolled trial involving septic humans (n=36) PMX hemoperfusion was able to improve blood gasses (after 96 hours) and Acute Physiology and Chronic Health Evaluation II score, reduce IL-8, plasminogen activator inhibitor-1, and neutrophil elastase (all after 48 hours post perfusion) [101]. PMX perfusion was also reported to reduce anandamide, a cannabinoid involved in induction of hypotension, in septic

human patients [102]. Showing mixed results, in an uncontrolled septic human trial (n=20) using PMX hemoperfusion, neutrophil elastase increased significantly immediately after perfusion but became reduced after 24 hours post perfusion and remained low to 72 hours [103]. A retrospective study found the removal of LPS by PMX contributed positively to cardiac output [104]. Others, however, have found PMX perfusion to not be efficacious in sepsis. Vincent, et al. reported a controlled experiment with 36 human sepsis patients found no statistically significant differences in the change in endotoxin or (IL)-6 levels or organ dysfunction (SOFA score). However, an increase in cardiac function was observed [97].

Lixelle beads, which were developed for the removal of β 2-microglobulin by hemoperfusion to treat dialysis-related amyloidosis, have been used in animal studies of endotoxemia as well. These beads have demonstrated a reduction in circulating cytokines, as well as some direct cytokine removal [105] and some hemodynamic benefits [106]. CTR beads, an adapted cellulose-based Lixelle bead with larger pores to adsorb more middle-weight molecules, have been shown to bind recombinant cytokines and enterotoxin *ex vivo*. Additionally, when endotoxemic rats were hemoperfused with CTR beads, the investigators noted reduced cytokines, improved hemodynamics, and reduced pulmonary neutrophil infiltration, and improved hemodynamics [107, 108].

To specifically remove bacteria and superantigens in sepsis, Fenwick, et al. perfused rats in a streptococcal shock model with a modified polystyrene filter and reported reduced bacterial counts [109].

In vitro attempts at targeting specific cytokines and autologous proteins using hemoperfusion have been made as well. These include TNF targeting with cellulose microparticles that were functionalized with monoclonal anti-TNF antibody or recombinant antibody fragments [110, 111] and specific protease targeting using different protease inhibitors (α 1-antitrypsin, pancreatic trypsin inhibitor, elastatinal and leupeptin) on membranes [112].

Others have combined renal replacement therapies such as high-flux dialysis [113] and push-pull pheresis [114-117] with adsorbents to increase efficacy of therapy. Hemoperfusion is often performed unintentionally, as adsorption onto the membrane was found to be the primary mechanism of observed cytokine reduction (e.g. IL-6) in membrane-based (AN-69, PMMA) hemofiltration [118-120].

The broad interest in hemoperfusion in sepsis as reviewed above attests to the potential of this type of therapy. Narrow spectrum hemoperfusion targeting of extracellular hemoglobin is particularly promising, as it may remove a dangerous, but largely overlooked mediator of sepsis severity.

Dissertation hypothesis and objectives

Plasma hemoglobin is dangerous during sepsis due to plasma hemoglobin's damaging oxidative effects, powerful stimulation of systemic inflammation, and increased lethality in the presence of bacterial endotoxin. It is often present in the circulation of septic patients in quantities unmanageable by passive immunization therapies. Although this danger has been recognized, the removal of plasma hemoglobin from the septic patient has not previously been considered. The hypothesis tested is that the extracorporeal removal of plasma hemoglobin in sepsis may be therapeutic.

To test the hypothesis, the following objectives have been set:

- A. To construct haptoglobin-bound beads to bind hemoglobin in solution
- B. To construct an apparatus for removing hemoglobin from solution
- C. To develop a kinetic model of hemoglobin removal under flow
- D. To explore therapeutic value of hemoglobin removal

Chapter 2: Adsorptive column for the removal of plasma hemoglobin (Objective A, B)

This chapter includes a description of the purification of haptoglobin from plasma and the manufacture, processing, and characterization of haptoglobin-beads. Description of the design, construction, and characterization of the

adsorptive column and miniaturized circuit for *in situ* filtration of rats are also included.

Chapter 3: Model of hemoglobin removal during hemoperfusion (Objectives C)

In this chapter, the relevant intrinsic properties of the column were measured and a kinetic model of hemoglobin removal based on a set of equations was developed. The application of this model accurately predicted experimental hemoglobin removal from saline and plasma, both *in vitro* as well as *in situ* in rats.

Chapter 4: Extracorporeal removal of plasma hemoglobin during sepsis decreases cytokine levels and attenuates organ damage (Objective D)

This chapter describes the immunological, physiological, and survival benefits of hemoglobin removal in sepsis. The dose-dependent lethality of elevated plasma hemoglobin in murine sepsis and the survival benefit of preventing its elevation through filtration were demonstrated. The proinflammatory underpinnings of hemoglobin-triggered sepsis severity were explored in cultured macrophages. The application of the therapy to rats *in situ* demonstrated the feasibility and therapeutic value of extracorporeal plasma hemoglobin removal in sepsis.

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Chapter 2

Adsorptive column for the removal of plasma hemoglobin

INTRODUCTION

Sepsis is a syndrome that is associated with multiple organ failure and significant morbidity and mortality. It affects 700,000 patients annually[1], is the 10th leading cause of death overall in the United States[2] and is rising in incidence[3]. The pathogenesis of sepsis is mediated by circulating toxins[4, 5], including extracellular hemoglobin. We have developed an extracorporeal technology whereby extracellular hemoglobin can be removed through direct absorption onto a bead column coated with haptoglobin. This chapter discusses the development of the beads, the column, and the circuitry involved in the application of this technology.

The Ligand: Haptoglobin

Mass Balance of Haptoglobin

One tetrameric haptoglobin molecule binds with two dimeric hemoglobin molecules (tetrameric hemoglobin will spontaneously dimerize in plasma), a stoichiometric factor of 1:1. On a mass per mass basis, 1 mg of haptoglobin, depending on the subunit makeup of the haptoglobin sample, binds to 0.3-0.6 mg of hemoglobin. Based on experiments enumerated below (Fig 2-14), 1 mg of bead-bound haptoglobin (Sigma) is capable of binding approximately 0.2 mg of hemoglobin. The concentration of plasma hemoglobin in septic rats is less than 50 mg/dL (Chapter 4, Figure 4-2a). To decrease plasma hemoglobin by 50mg/dL, 25.8 mg of haptoglobin is required:

$$\left(50 \frac{\text{mg}_{-}\text{Hemoglobin}}{\text{dL}} * 5 \frac{\text{mg}_{-}\text{Haptoglobin}}{\text{mg}_{-}\text{Hemoglobin}} * 0.103\text{dL} \right) = 25.8\text{mg}_{-}\text{Haptoglobin}.$$

MATERIALS AND METHODS

Haptoglobin Purification

To purify haptoglobin for use in experiments, fifteen liters of human plasma was collected under NSLIJHS IRB protocol # 06-03-049 from unused autologous donations. Plasma from multiple donors was pooled and centrifuged to pellet any formed elements remaining in the bag. This was important, as any red blood cells that lyse during plasma storage at -20°C will spill hemoglobin into the plasma, interfering with the purification process. The plasma supernatant was then aliquoted and stored at -20°C.

Bench-scale purification

The purification technique used was based on the procedures published by Eurell et.al. [6] and Liao et.al. [7].

Haptoglobin Capture

Four mL of hemoglobin-bound beads (hemoglobin-beads; Sigma) were added to a 15 mL column and drained. An HCl-acetone solution was added to the column to strip the heme from the hemoglobin-beads. The literature [6] noted that removing the heme prior to exposure to plasma will reduce heme-binding impurities such as hemopexin. The beads were washed extensively with the HCl-acetone solution until the eluate ran clear. The same procedure was performed simultaneously without removing heme. In that column, water was used to rinse the beads instead of HCl-acetone. The beads were then equilibrated by rinsing 7 times with 4 mL of binding buffer (4x binding buffer= 0.01 M Tris-HCl, 5M NaCl, pH 7.0). Forty mL of plasma was thawed and centrifuged at 3000g to pellet any

precipitate. The plasma was transferred to a fresh tube and diluted 25% in 4x binding buffer. The plasma solution was then slowly added to the hemoglobin-bead column to bind haptoglobin from solution. The flow-through was collected and added back to the column two additional times.

Haptoglobin Elution

The column was then rinsed extensively in binding buffer to remove nonspecific plasma elements. The flow-through was assayed visually using a photometric dye (Biorad Protein Assay) and the column was rinsed until no protein was detected. Elution of protein was performed using increasing concentrations of guanidine elution buffer (guanidine in binding buffer); 0.8 M, 1.6 M, 3.5 M, and 5 M guanidine. Each eluate was collected into a separate tube. For fast detection of protein, the guanidine was removed from a 70 uL sample of each eluate using Micro Biospin columns (BioRad). The samples were then prepared in Laemmli buffer containing β -mercaptoethanol and electrophoresed through Tris-HCl gel (BioRad). The protein was then visualized using GelCode Blue stain (Pierce), a nonspecific protein stain.

Haptoglobin Concentration, Guanidine Removal

The guanidine elutions were pooled and concentrated roughly 10-20 times using Amicon Ultra centrifugal filters with a molecular weight cutoff of 10 kDa (Millipore). The haptoglobin concentrate was then dialyzed into PBS using Slide-A-Lyzer dialysis cassettes with a molecular weight cutoff of 7 kDa (Pierce) until the guanidine concentration was less than 10^{-5} mM.

Haptoglobin Quantification

The concentration of purified protein was quantified using a spectrophotometric assay. Briefly, 10 μ L of diluted sample was added to 200 μ L dye (Biorad Protein Assay), read at 595 nm, and interpolated from a standard curve created with bovine serum albumin (BSA).

Purification of Haptoglobin; Scaled up (>100 mL beads)

Column packing

Hemoglobin beads were packed in a 30 cm column (BioRad) with an ID of 2.5 cm (4.9 mL/cm). Briefly, the bead slurry was poured into the column and positive pressure applied to pack the beads by sealing the top with a flow adaptor and using a programmable pump (BioRad Econo Gradient Pump) to force air into the column. Before the packed bead surface was exposed to air, PBS was pumped in to equilibrate the column. A flow adaptor was pressed down to the bead-PBS interface and any air bubbles carefully removed. The beads were further packed and equilibrated by pumping PBS until the eluate became clear. The beads were then washed in 5 M guanidine in haptoglobin binding buffer to remove any loosely bound hemoglobin from the beads (to minimize hemoglobin in the haptoglobin elution). The buffer was then exchanged for haptoglobin binding buffer by pumping through 4 column volumes of 1X haptoglobin binding buffer at 3 mL/min.

Haptoglobin Capture

A reservoir of 200mL human plasma prepared was then pumped once through the column at 2 mL/min.

Haptoglobin Elution

The haptoglobin was eluted using a stepwise gradient of guanidine in haptoglobin binding buffer and assayed for haptoglobin concentration and purity as above. The elution was captured in 6 mL fractions using a synchronized fraction collector (BioRad model #2128). Elution buffer concentration was controlled by a program-controlled mixer (BioRad) using source solutions of 1X haptoglobin binding buffer and 5M guanidine. Protein concentration within the fractions was assayed using a spectrophotometric assay (BioRad protein assay). Buffer was exchanged in selected fractions using Micro Bio-Spin columns (BioRad) and protein assayed by SDS-PAGE and GelCode protein staining (Pierce).

Haptoglobin Concentration, Guanidine Removal

The guanidine elutions containing purified haptoglobin were pooled and concentrated using Amicon Ultra centrifugal filters with a molecular weight cutoff of 10 kDa (Millipore). The haptoglobin concentrate was then dialyzed into PBS using Slide-A-Lyzer dialysis cassettes with a molecular weight cutoff of 7 kDa (Pierce) until the guanidine concentration was less than 10^{-5} mM.

Haptoglobin Quantification

The concentration of purified protein was quantified using a spectrophotometric assay. Briefly, 10 uL of diluted sample was added to 200 uL dye (Biorad Protein Assay), read at 595 nm, and interpolated from a standard curve created with bovine serum albumin (BSA).

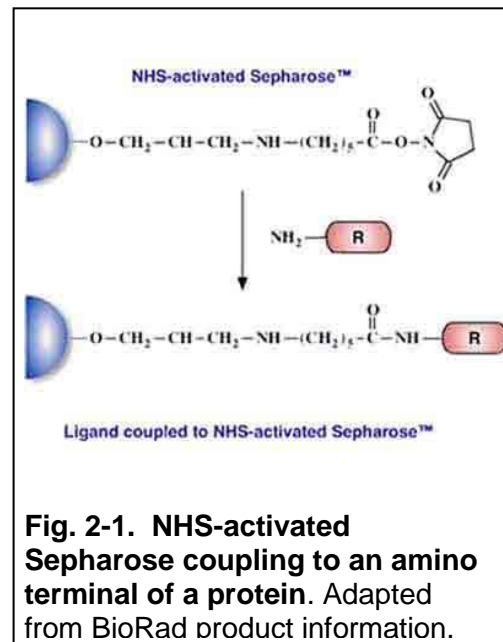
Reduction of Apolipoprotein A1 in haptoglobin preparations

To reduce apolipoprotein A1 contamination in haptoglobin preparations, haptoglobin was bound to hemoglobin-beads and washed as described above. The haptoglobin was eluted with glycine-HCl at pH 3 as described [8] and collected in fractions. The protein was then visualized as above using GelCode Blue stain.

A second method to selectively remove apolipoprotein-A1 was developed. The guanidine-eluted fractions were concentrated using Amicon filters with a molecular weight cut-off of 50 kDa. The apolipoprotein-A1 passed through the filter while the haptoglobin was retained.

Regeneration of hemoglobin-column

Haptoglobin was purified from human plasma as described above. The hemoglobin-column was regenerated by washing in guanidine (5M) until no protein is detected at the outlet. The column was then washed in 1X haptoglobin-binding buffer. Haptoglobin yield was measured after all fractions had been combined and concentrated. Protein was measured by spectrophotometric assay (Biorad). A linear slope was fitted to the data in Fig. 2-10.



The Beads

N-hydroxysuccinimide (NHS)-Activated Sepharose Fast Flow 4 Beads (Amersham Bioscience) were selected. These beads have a mean diameter of

90 μm , ranging from 45-165 μm , and are formed from 4% crosslinked agarose). The beads are coupled with a 6-aminohexanoic acid spacer. The terminal carboxyl group is activated by esterification with NHS. This active ester will couple to primary amino groups of ligands forming a stable amide linkage (Fig. 2-11).

Binding of haptoglobin to the sepharose beads.

The manufacturer's instructions were followed:

- a) One mL of beads was washed twice with 14X volumes of cold 1mM HCl.
- b) Five mg of human haptoglobin (Sigma cat # H3536, lyophilized powder) were suspended in 1 mL cold phosphate buffered saline (PBS) and added to the beads. The binding reaction took place overnight at 4°C.
- c) The beads were transferred to a BioRad Poly-Prep chromatography column (cat # 731-1550); the PBS solution was gravity drained; and the filtrate was collected and protein quantified using a Bradford assay. The beads were washed three additional times with PBS. The washes were tested for protein and all unbound protein quantified.
- d) The remaining sites on the beads were neutralized by incubating them in a 100 μM Tris-HCl solution (pH 7.2) for 2 hours at room temperature.
- e) The beads were rinsed extensively using alternating buffers of 50 μM Tris-HCl at pH 8.0 and 50 μM Sodium Acetate at pH 3.5 alternated 3 times. They were then suspended in an isotonic PBS solution at a pH of 7.0. Control beads were prepared in an identical manner, except that no haptoglobin was added to the binding reaction in step b. Haptoglobin bound to the column was calculated as

the amount of hemoglobin added to the active beads minus the total of unbound haptoglobin measured in the filtrates.

Determination of the optimum binding concentration of haptoglobin with respect to the hemoglobin-binding capacity.

Haptoglobin-beads were manufactured by varying the initial concentration of native purified haptoglobin exposed to activated beads. Hemoglobin-binding capacity for each batch of haptoglobin-beads in a batch reactor was measured as described in Chapter 4.

Preparation of beads for blood contacting

Sterilization

The ethanol concentration needed to sterilize the haptoglobin-beads while retaining at least 80% of the hemoglobin binding capacity was determined to prepare the haptoglobin beads for in vivo use. Haptoglobin-beads were immersed for varying times in ethanol concentrations between 10% and 70% in normal saline followed by robust washing with sterile saline. Sterility was demonstrated by incubating overnight at 37°C in Luria-Bertani (LB) bacteria growth medium and checking for growth, as measured by turbidity. The tubes were then stored for six weeks at room temperature and checked again for growth. Sterile LB medium served as control. Hemoglobin removal capacity of the sterile haptoglobin-beads was then assessed by exposing the sterile beads to a 50 mg/dL hemoglobin solution in saline.

LPS Removal

The optimal method to neutralize endotoxin on the haptoglobin-beads was determined. Haptoglobin-beads were prepared as above and added to 1.5 mL tubes. Polymyxin (3000U/mL), Triton X-114 (1:20), and PyroCLEAN (standard means to remove LPS), as well as saline (control) were added to respective tubes, which were then incubated with agitation for 1 hour. The tubes were then centrifuged for 5 minutes at 4000g and the supernatant discarded. The beads were then washed 5 times with endotoxin-free water.

To assess any reduction in hemoglobin-binding activity, 40 uL of the beads were placed in a tube containing 300 uL of 50 mg/dL hemoglobin in PBS and incubated under gentle agitation for 30 minutes. As described above, the tubes were centrifuged and the supernatant measured spectrophotometrically. The decrease in hemoglobin concentration was compared to the decrease in hemoglobin concentration from the saline-washed bead supernatant. The LPS on the cleaned and the uncleaned beads was assessed using a quantitative Limulus Amoebocyte Lysate (LAL) assay.

Depletion of haptoglobin from plasma

Haptoglobin was depleted from plasma for use in *in vitro* hemoglobin-binding studies. Plasma that flowed through the hemoglobin-column during haptoglobin purification described above was retained. Haptoglobin was measured by western blot (goat anti-human haptoglobin primary antibody, MP Biomedicals).

Functionality Tests in Blood

We have seen above that haptoglobin-beads bound hemoglobin in a saline solution. Plasma, on the other hand, is a biologically active fluid, containing proteases and other potentially disruptive molecules and hemoglobin uptake may be reduced by exposure to plasma, through lysis, leaching, denaturation, or surface fouling. The hemoglobin-binding capacity of haptoglobin-beads in plasma and retention of that capacity after one hour incubation was determined. Haptoglobin- and control- beads (n=3/group) were suspended in haptoglobin-depleted plasma (human) with no significant hemoglobin content (≤ 3 mg/dl) for an hour at 37°C, which was the length of the hemoperfusion procedure. The tubes were centrifuged for 5 minutes at 4000g to pellet the beads and the supernatants were removed. The beads were washed and suspended in saline or plasma spiked with 100 mg/dL hemoglobin. After another incubation of 30 minutes, the tubes were centrifuged again and the supernatant removed. The concentration of hemoglobin in the supernatant was assayed spectrophotometrically as above. The difference in hemoglobin concentration between the supernatant from the haptoglobin-beads and the supernatant from the control- beads represented the quantity of hemoglobin that was removed from solution by the haptoglobin-beads.

To directly visualize the hemoglobin bound to the beads, the beads were washed extensively in saline and boiled in Laemmli containing β -mercaptoethanol for 5 minutes to denature and solubilize all the bound protein. The supernatant was electrophoresed through a Tris-HCl gel and stained with GelCode Blue protein stain.

Functionality test of purified native haptoglobin-beads

The hemoglobin-binding activities of haptoglobin-beads composed of Sigma hemoglobin and purified native haptoglobin were compared. Control beads and haptoglobin-beads originating with purified native and Sigma haptoglobin source material

were suspended in 50% slurry in PBS. One hundred μL aliquots of the suspensions were each added to 3 1.5 mL centrifuge tubes. One hundred and fifty μL of a 200 mg/dL hemoglobin saline solution was added to each tube and the tubes gently shaken on a touch-mixer to maintain the beads in suspension. At 20 minutes the tubes were centrifuged to pellet the beads. A sample of the supernatant was withdrawn and the hemoglobin concentration

assayed spectrophotometrically as above. This test was performed on beads containing purified native haptoglobin after 0, 3, and 11 weeks at 4°C in 50% ethanol.

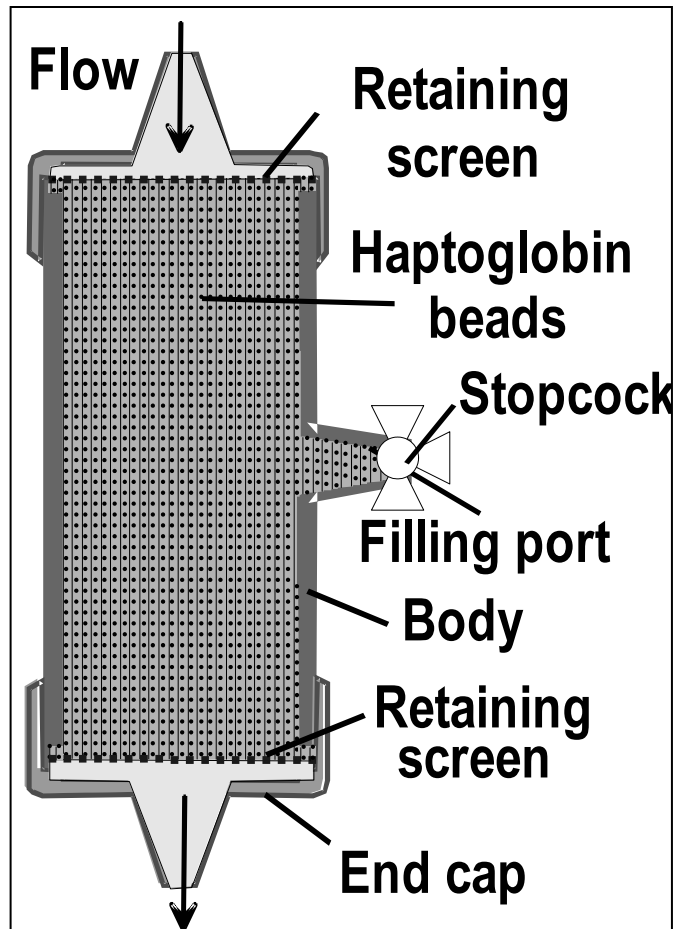
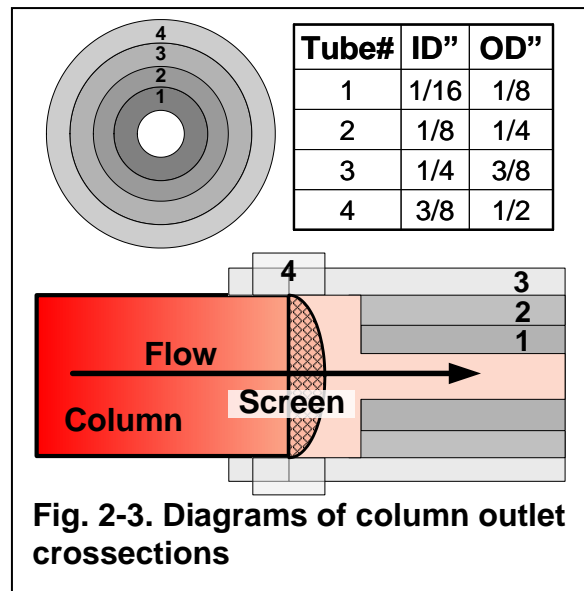


Fig. 2-2. Column was constructed. Columns were manufactured with a luer-fitted $\frac{1}{4}$ " ID connector bounded on each end with a 37 μm mesh screen. Endcaps were constructed of nested tubing and the column was filled with beads (Fast Flow 4 agarose).

Column design

To remove hemoglobin under flow conditions, a column was designed to expose and retain the haptoglobin-beads to solution. The column assembly (Fig. 2-2) retained the beads while allowing blood to flow. The body of the housing incorporated a filling port that allowed the introduction of the beads, here a 1/4" inner diameter (ID) Luer-containing perfusion connector ended with 3/8" ID Tygon 3606 (Polyvinyl chloride, PVC) tubing. A polyester screen with 37 μ m diameter pores and 31% open area (commonly used in arterial line filters during cardiopulmonary bypass) was placed at the inlet and outlet of the body and

retained the beads while allowing unhindered blood flow (Fig. 2-3). To ensure that the screen was supported, the screens are adhered using UV-cured polyurethane to the 1/4" ID PVC tube, with a wall thickness of 1/16". In addition, a second segment of PVC of equal dimension (3/8" ID, 1/2" OD)



was pressed tightly against the screen and bonded in place as above. The screens were prevented from contacting the end caps through the inclusion of void space within the end cap, allowing for the screen to slightly deform in the direction of flow without contacting any obstructing surface. The inlet and outlet were assembled by nesting increasing ID (1/16", 1/8", and 1/4") PVC tubing, cut flush, and inserted into a 3/8" ID sleeve (Fig. 2-3). The tubes were secured to the

body of the column housing by solvent bonding all interfaces with cyclohexanone. To increase hemocompatibility, heparin-coated screens were used. The filling port is used to load sterile column media at the onset of filtration.

Fluid mechanics through column

Homogeneous flow is required to minimize error due to variable flow through the column. Flow was observed in two ways: transcolumn pressure measurements (pressure at inlet- pressure at outlet) to ensure predicted resistance to flow through the columns and visual observation of hemoglobin binding in the column. Plasma (from heparin-treated human blood) or hemoglobin solution (50mg/dL saline) were pumped (0.6, 1 mL/min; Econo Gradient Pump, BioRad) in a single pass through variably packed columns (0.5, 1.5 mL) with pressure transducers at the column inlet and outlet. Pressure data was recorded at 2 Hz using a MP150 system with TSD104A pressure transducers and running AcqKnowledge software (Biopac Systems, Goleta, CA). Calculated transcolumn pressure, ΔP (ΔP = inlet pressure- outlet pressure), was compared to the transcolumn pressure predicted by the Ergun equation for pressure through a packed column:

$$\Delta P = \frac{150 \mu v' L}{D_S^2} \frac{(1 - \epsilon)^2}{\epsilon^3} + \frac{1.75 \rho v'^2 L}{D_S} \frac{(1 - \epsilon)}{\epsilon^3} \quad (1)$$

where, μ is dynamic viscosity, ρ is density, ϵ is void fraction, v' is the superficial velocity, L is the column height, and D_S is the bead diameter[9]. ϵ in the column was about 0.36, in agreement with published values for ϵ with an average random packing of spheres[10, 11]. Column ΔP was predicted based on values

listed in Table 2-1. Hemoglobin-binding to the haptoglobin-beads in the column were video recorded under flow and the uniformity of flow determined by visual observation of bead reddening.

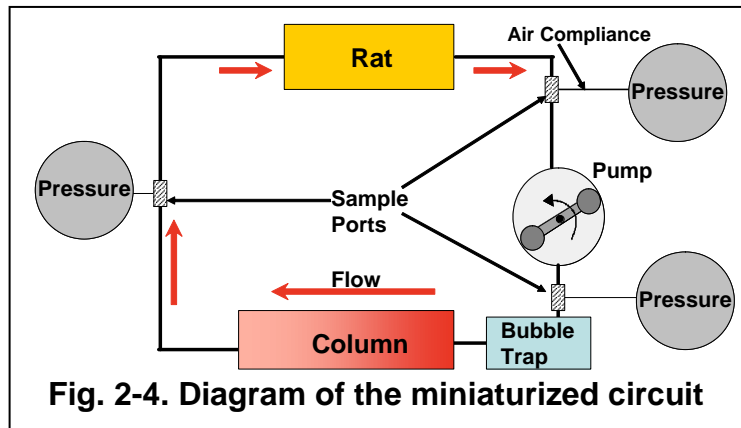
Table 2-1. Values of column parameters for volume=1.5 mL, flow=1 ml/min

| Parameter | Symbol | Value | Units |
|----------------------|-----------------|---------------------|-------------------|
| Column Diameter | D_c | $6.4 \cdot 10^{-3}$ | m (1/4 inch) |
| Column Height | L | $4.7 \cdot 10^{-2}$ | m |
| Void fraction | ϵ | 0.36 | |
| Superficial velocity | v' | $5.3 \cdot 10^{-4}$ | m/s |
| Bead diameter | D_s | $9.0 \cdot 10^{-5}$ | m |
| Dynamic viscosity | μ (plasma) | $1.6 \cdot 10^{-3}$ | Kg*m/s |
| Dynamic viscosity | μ (blood) | $4.0 \cdot 10^{-3}$ | Kg*m/s |
| Density | ρ (plasma) | 1025 | kg/m ³ |
| Density | ρ (blood) | 1060 | kg/m ³ |

Circuit design

To remove hemoglobin from rats in vivo, a miniaturized circuit was developed, prototyped and tested. The circuit (Fig. 2-4) was composed of the column, pump, interconnecting tubing (PVC as used in dialysis circuits), pressure monitors, a bubble trap, and sampling ports. The column has been described above.

The interconnecting tubing had an ID of 1/32", which allows for a small priming volume, minimizing the volume of blood external to the rat. The pump raceway contained larger 1/16" ID tubing to minimize hemolysis [12].



The bendability of the material allowed for circuit compactness, which also

helped to minimize the priming volume of the circuit. In addition, bubbles were easily visualized and tracked through this clear material.

A Stockert-Shiley roller pump that had its occlusivity calibrated to minimize hemolysis [13] was used to circulate the blood through the circuit.

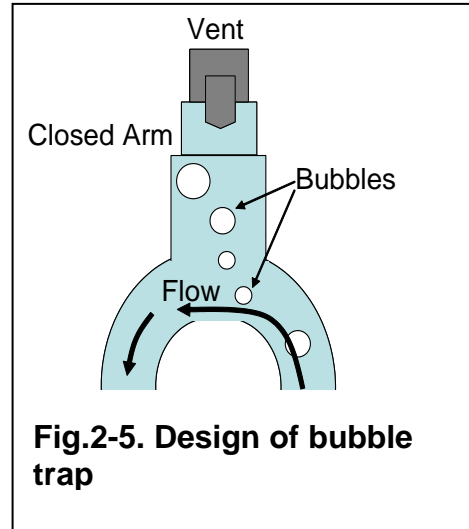
The pressures at the cannula outlet, the column inlet and the inlet of the return cannula were continuously monitored. The pressure at the cannula outlet was used to ensure that the pressure at the cannula tip remained positive. Maintaining positive pressure prevented vessel damage by withdrawing blood passively from the artery, which ensured that the vessel wall did not collapse upon the cannula. Had negative pressure at the cannula outlet been detected, the pump was temporarily shut off, the cannula adjusted, and the pump restarted.

To minimize the chance of flow obstruction due to the pulsatile nature of the roller pump, compliance was incorporated between the inlet cannula and the pump. Compliance was provided by air-filled tubing between the blood path and the pressure monitor. A saline-filled tube was placed between the blood path and the compliance to preclude air from the compliance from entering the circuit. Compliance to minimize pressure fluctuations on the column was provided by the air enclosed in the bubble trap (described below).

The pressure monitors between the pump and the column and between the column and the rat measured the pre- and post-column pressures, respectively. The difference between these pressures reflected the resistance across the column, which impacted the shear forces that are generated within the column. An increase in pressure difference can indicate clotting, bead

compaction, or entrapped air within the column. Tubing connecting the pressure monitors with the blood path were filled with sterile saline to provide accurate readings and prevent coagulation of entrapped blood.

Air bubbles within the circuit are detrimental for multiple reasons. Foremost among them are the effects they have on fluid flow through the column, including increasing shunting of blood away from the beads, and increasing the pressure drop across the column. The circuit had therefore been designed to incorporate a bubble trap to remove bubbles. Air is routinely



removed from clinical extracorporeal circuitry by taking advantage of the buoyancy of air within a region of relatively low velocity. Air within this circuit is difficult to trap, as bubbles flowing through the 1/32" ID circuit are dragged by a 1 mL/min flow (3.4 cm/sec). The purpose of the bubble trap (Fig. 2-5), therefore, was to decrease the velocity and the velocity-dependent fluid drag force of the blood to allow the buoyancy force on bubbles time to lift those bubbles to the surface of the tube. The bubble trap was formed by a connector that was designed to provide a blood path with increasing ID, the largest being 1/4" ID. The blood velocity decreased by the square of the ratio of the incoming and the bubble trap diameter. The trap directed blood flow up against gravity and through a bend. At the apex of the bend, a closed arm trapped bubbles that rose to the top of the channel. The air was removed via a release valve in the closed

arm.

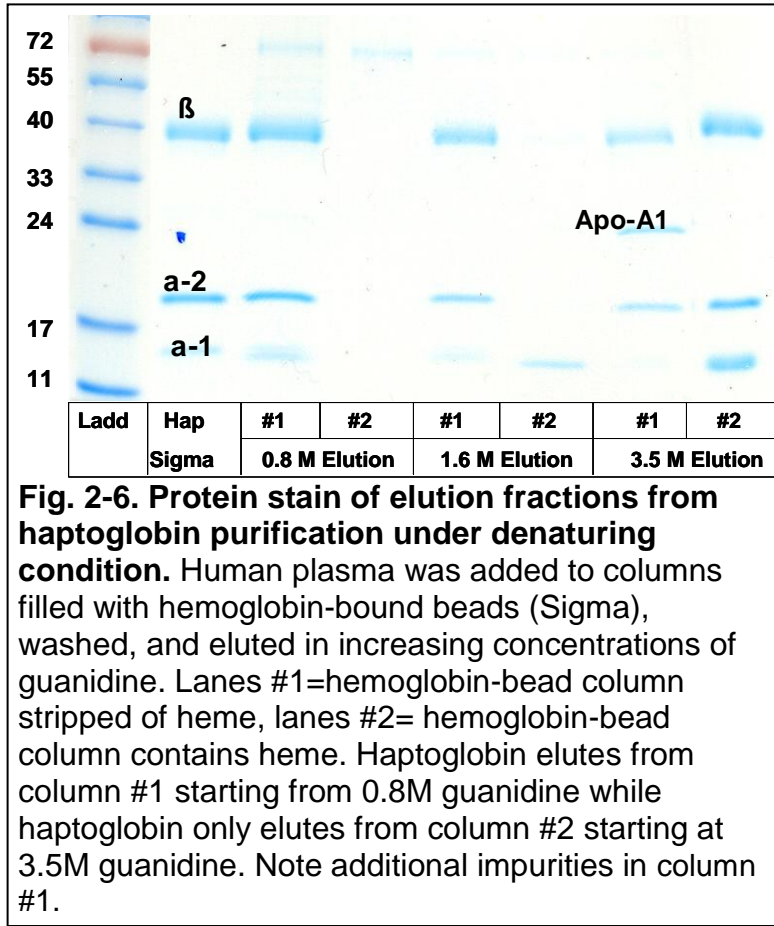
It was important to track and adjust for any drift over time in blood flow through the circuit. Blood flow was quantified periodically by injecting a small amount of air (0.05-0.1 cm³) and timing the bubble's transport through a demarcated 20 cm stretch of 1/32" ID tubing immediately upstream of the bubble trap.

Sampling ports were included in the circuit to allow for the analysis of blood across the pump and the column. Sampling ports were placed directly in the blood path to minimize blood volume required per sample, as well as to reduce the static blood volume within the port. The ports consisted of four-way stopcocks that were opened to allow blood to flow out of the circuit. After sampling, the stopcock was closed, flushed with saline, and wicked dried to prevent dilution of any subsequent sample. The sampling ports were also used to prime the circuit with sterile saline and to remove air from the circuit. The complete circuit, including the column housing without beads, was sterilized with ethylene oxide.

RESULTS

Hemoglobin-beads pretreated with HCl-acetone yielded decreased haptoglobin purity in bench-scale purification.

The image in Figure 2-6 shows the protein that has eluted off the hemoglobin-beads at various concentrations of guanidine. Note that all the elutions marked #1 and the 3.5 M elution marked #2 were fairly pure (in comparison to Sigma purified haptoglobin). Additionally, minimal haptoglobin eluted off of



the hemoglobin-beads that contain heme until the 3.5 M guanidine elution. This allowed for a more purified product, as any protein bound to hemoglobin-beads or to the haptoglobin itself with a lower binding strength can be removed before any significant haptoglobin elutes. After rinsing the beads thoroughly in 3.5 M guanidine, no additional protein is eluted in 5 M guanidine. Eleven to 12 mg haptoglobin were purified/ 4 mL of beads and 21 mL plasma, which is similar to the published yields [6].

Haptoglobin was purified in scaled-up procedure and apolipoprotein-A1 coeluted.

As seen in Figure 2-7, haptoglobin is eluted from the column at 3.5 M guanidine. Apolipoprotein-A1 co-elutes under these conditions toward the

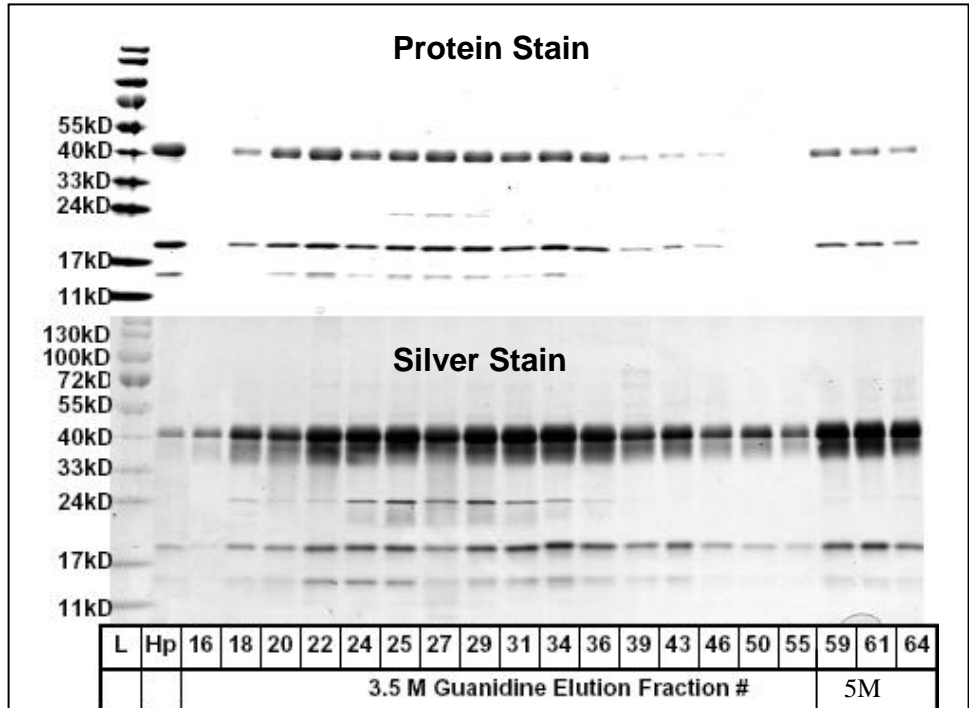


Fig. 2-7. Protein (top) and silver stain (bottom) of column elution fractions from haptoglobin purification. Elution fractions of 3.5 and 5 M guanidine were collected, buffer exchanged, and analyzed after denaturing SDS-PAGE. Purification yielded a high degree of haptoglobin purity, especially in fractions >#36. Fractions 59-64 were eluted in 5M guanidine.

early stage of the elution, and is almost completely removed by fraction #36. Spectrophotometric quantification of the concentrated and dialyzed eluate fractions showed approximately 100 mg of haptoglobin purified from a single pass of 160 mL of plasma.

Apolipoprotein-A1 contamination is not removed with Glycine-HCl elution

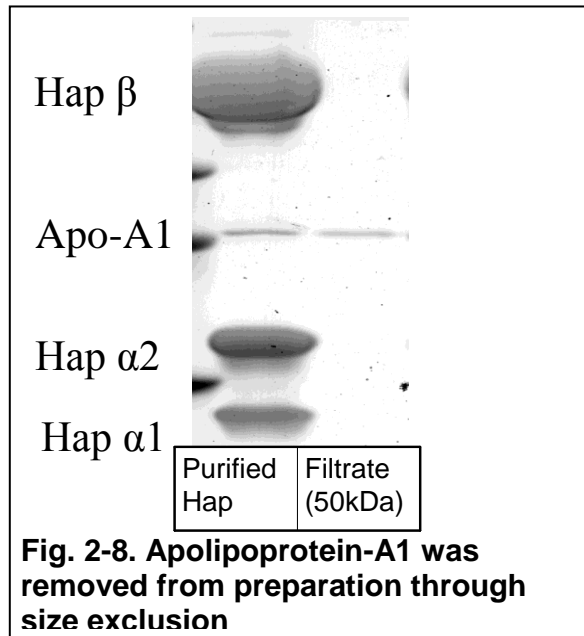


Fig. 2-8. Apolipoprotein-A1 was removed from preparation through size exclusion

We attempted to elute haptoglobin from hemoglobin-columns using acidic Glycine-HCl elution to minimize Apolipoprotein-A1 contamination. A large quantity of haptoglobin coeluted with apolipoprotein A1 (fractions #5-12; Fig. 2-9).

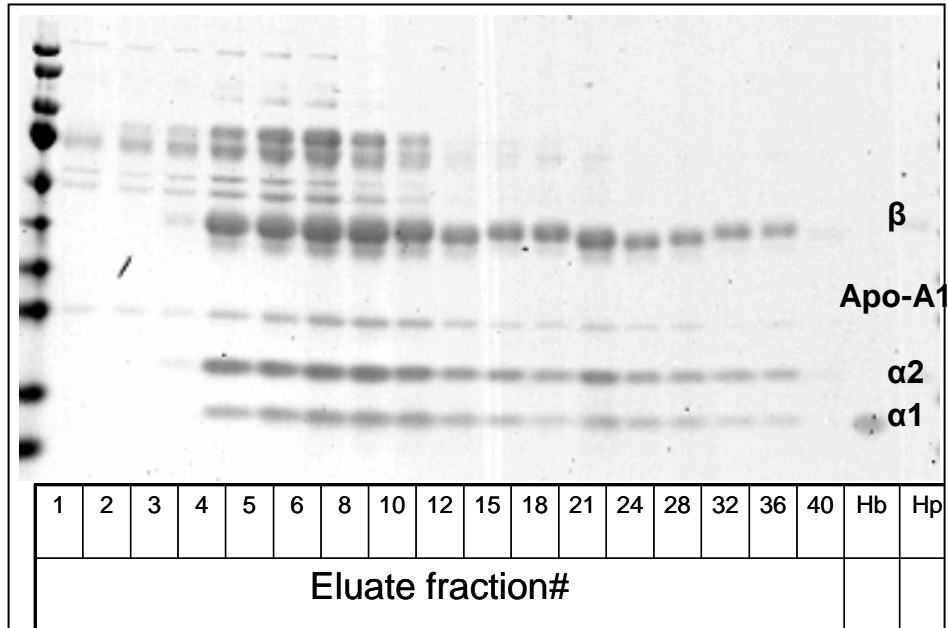


Fig. 2-9. Protein stain of column elution fractions from haptoglobin purification. Glycine-HCl elution fractions were collected and analyzed after denaturing SDS-PAGE. Acidic elution resulted in co-elution of impurities.

Purification of haptoglobin: Regeneration of hemoglobin-column

There was a decline in haptoglobin yield by approximately 20 mg/cycle ($R^2=0.94$).

Attachment of haptoglobin to the sepharose beads

The protein assay of the beads' filtrate (after the binding step) measured 50µg/mL. The assay of the first wash revealed 25µg/mL of protein, and no protein was detected in the filtrate of the final 2 washes. Since the amount of unbound haptoglobin was quantified, the amount of haptoglobin that bound to the 1 mL of beads was calculated to be 4.9 mg.

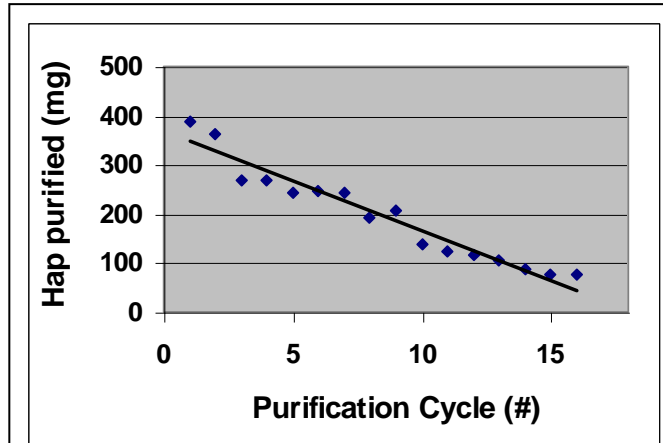


Fig. 2-10. Hemoglobin-column loses haptoglobin-purification capacity when regenerated.

Optimize binding concentration of haptoglobin with respect to the hemoglobin-binding capacity.

Haptoglobin-beads with a haptoglobin content of 8.4, 14.6, 15.9, and 18.8 mg haptoglobin/ mL bead exhibited hemoglobin binding of 2.5 ± 0.2 , 3.5 ± 0.1 , 3.6 ± 0.1 , 3.5 ± 0.1 mg/mL bead respectively (Figure 2-11). Data presented as

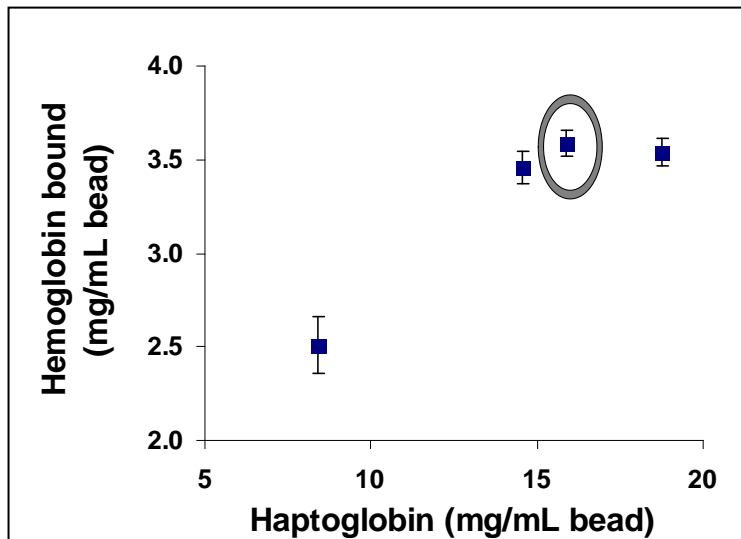


Fig. 2-11. Haptoglobin binding to beads was optimized. Haptoglobin-beads of varying haptoglobin content were manufactured and tested for hemoglobin binding capacity. Optimum concentration determined at 16 mg/mL bead. Data presented as mean \pm SEM; n=3.

mean \pm SEM; n=3.

Preparation of beads for blood contacting

Sterilization of the beads

Beads exposed to 50% ethanol for 24 hours or 70% ethanol for at least 5 minutes and incubated in LB media for 6 weeks, exhibited no growth and were considered sterile (Fig. 2-12a). The

hemoglobin-binding capacity of the sterilized beads is shown in Fig. 2-12b as a percentage of the hemoglobin-binding capacity of the beads not exposed to ethanol. The hemoglobin binding capacity of the beads

exposed to 50% ethanol for 24 hours was $96\pm 1\%$ and that of the beads exposed to 70% ethanol for 5 minutes was $86\pm 1\%$.

LPS Removal

| EtOH | Incubation Time | | |
|------|-----------------|--------|---------|
| | 5 Minute | 3 Hour | 24 Hour |
| 35% | | | |
| 50% | | | Sterile |
| 70% | Sterile | | |

Fig. 2-12a. Sterility of beads under various conditions. Beads were incubated in ethanol for various lengths of time before equilibrating in saline. Sterility was assessed by incubating in bacteria growth medium and checking for turbidity. Beads were sterilized in 50% ethanol for 24h and 70% ethanol for at least 5 min.

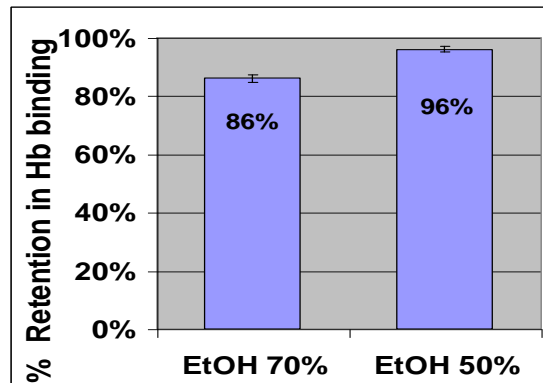


Fig. 2-12b. Hemoglobin binding capacity of sterilized beads. hemoglobin-binding was assessed for haptoglobin-beads under minimal sterilizing conditions. There was minor loss in activity when sterilized in 70% ethanol and minimal loss in activity when sterilized in 50% ethanol.

As depicted in Fig. 2-13, the hemoglobin-binding activity of the haptoglobin beads washed with polymyxin or with Pyroclean was undiminished, relative to the binding activity of the control haptoglobin beads washed with saline. Triton X-114 reduced the binding activity of the haptoglobin beads by 11%. All three LPS-neutralizing washes reduced LPS activity below 0.1 U/mL by LAL assay from the 61 U/mL on the saline-washed beads. That level of LPS activity translates into less than 0.01 ng LPS / mL bead, which was deemed negligible.

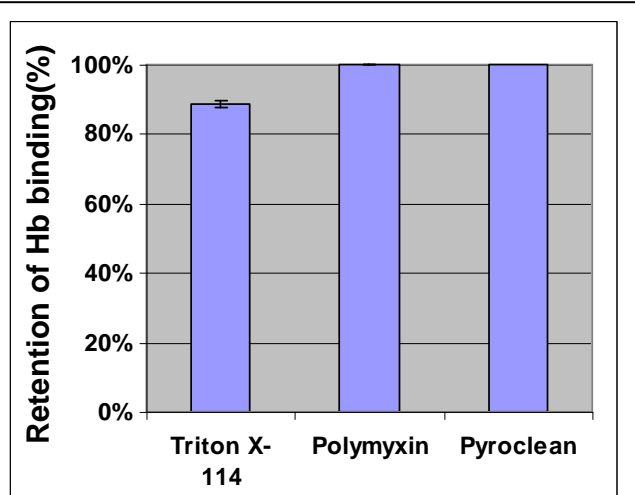


Fig. 2-13. Percent retention in hemoglobin-binding following LPS removal. Hemoglobin-binding capacity of haptoglobin-beads exposed to endotoxin-deactivating compounds was quantified. Polymyxin and Pyroclean treatment did not affect hemoglobin-binding capacity.

Depletion of haptoglobin from plasma

There was no detectable haptoglobin in the haptoglobin-depleted plasma (Fig. 2-14).

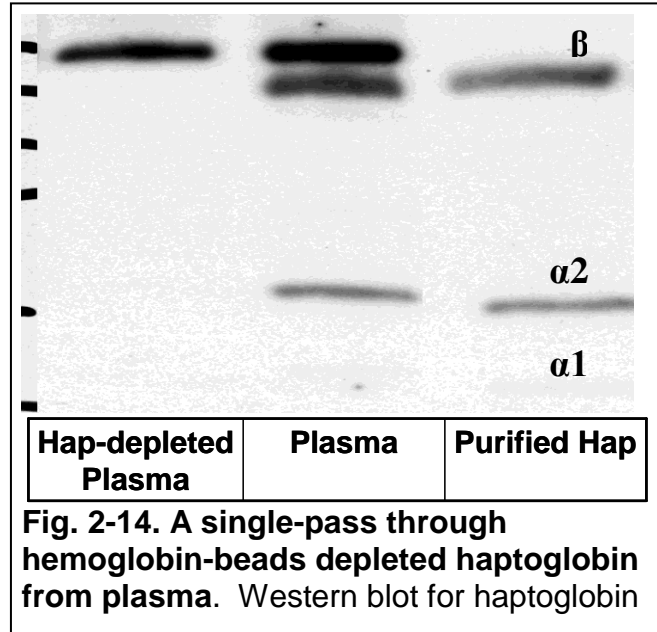
Hemoglobin-binding capacity of haptoglobin-beads in plasma and retention of that capacity after one hour incubation.

As shown in Figure 2-15a, there was no significant difference in hemoglobin-binding capacity between any of the groups tested. All haptoglobin beads bound hemoglobin from spiked plasma or saline at about 0.2 mg hemoglobin/mg haptoglobin. Figure 2-15b shows that there was significant

binding of hemoglobin on haptoglobin beads when compared to the hemoglobin bound to control- beads.

Comparison of the hemoglobin-binding activity of haptoglobin-beads composed of Sigma hemoglobin vs. purified native haptoglobin

The purified native haptoglobin-beads exhibited a hemoglobin-binding capacity of 0.35 ± 0.02 mg hemoglobin/ mg haptoglobin while the Sigma haptoglobin-beads exhibited a hemoglobin-binding capacity of 0.22 ± 0.01 mg hemoglobin/ mg haptoglobin and was not significantly different 3 or 11 weeks later, see Fig. 2-16.



Flow through the sorption column was uniform

Plasma was pumped through the columns and pressures measured. Relative to the predicted pressure the transcolum pressures for 1.5 mL columns were $96 \pm 6\%$ and $101 \pm 4\%$ at 1.0 and 0.6 mL/min, respectively ($p=0.3$, 0.9 by paired t-test, respectively). The transcolum pressure for the 0.5 mL column was $104 \pm 9\%$ at 1.0 mL /min, ($p=0.9$ by paired t-test; Figure 2B). Taken together, the actual pressure was $103 \pm 3\%$ of that predicted by the Ergun equation ($p=0.4$ by paired t-test). Hemoglobin solution was pumped through the column. The white haptoglobin-beads in the column reddened in a radially symmetric pattern that

moved axially from the inlet to the outlet of the column. No channeling between the beads was observed.

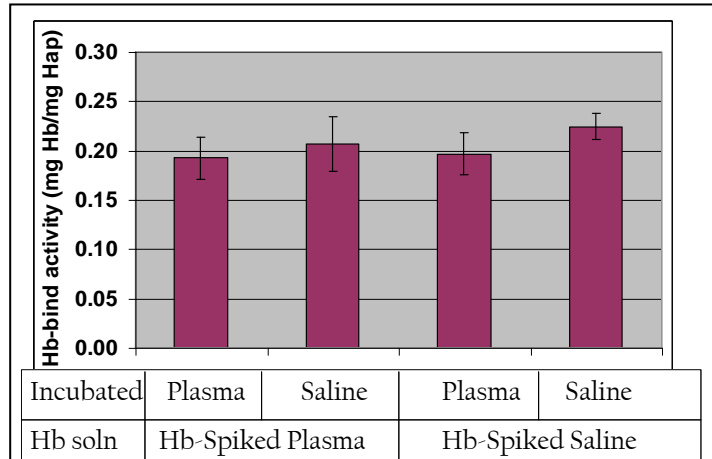


Fig. 2-15a. The capacity of haptoglobin-beads to bind hemoglobin in saline and plasma. Haptoglobin-beads were incubated for 1hr in plasma or saline, then transferred to either plasma or saline spiked with 100 mg/dL hemoglobin. The hemoglobin-binding capacity of haptoglobin-beads was determined. There was no significant reduction in hemoglobin-binding capacity due to incubation in plasma.

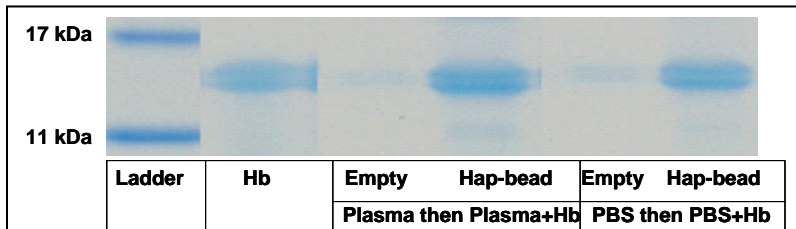


Fig. 2-15b. The capacity of haptoglobin-beads to bind Hb in saline and plasma. Bound protein was stripped from beads in Laemmli buffer, separated by SDS-PAGE, and visualized with a protein stain. Haptoglobin-beads bound significantly more hemoglobin than did empty beads.

DISCUSSION

The bench-scale haptoglobin purification was successful.

Performed either with or without heme on the hemoglobin-beads, a high purity haptoglobin product was obtained. The major contaminating

protein was apolipoprotein A-I (Fig 2-6) which is a haptoglobin binding protein [8,

14]. The presence of apolipoprotein may influence haptoglobin-hemoglobin binding [8], so means for its exclusion (such as glycine-HCl elution) were developed. The more obstructive contaminant, especially in the heme-containing hemoglobin-bead product, is hemoglobin itself. This

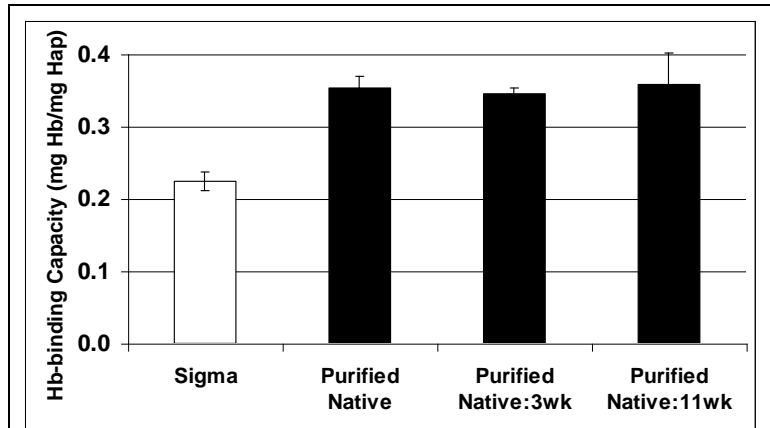


Fig. 2-16. Hemoglobin-binding capacity of haptoglobin-beads; Sigma vs. native purified. Haptoglobin purified in-house was attached to beads and Hb-binding capacity quantified in batch reaction. When attached to beads, purified native haptoglobin demonstrated 60% greater hemoglobin-binding capacity than purchased haptoglobin.

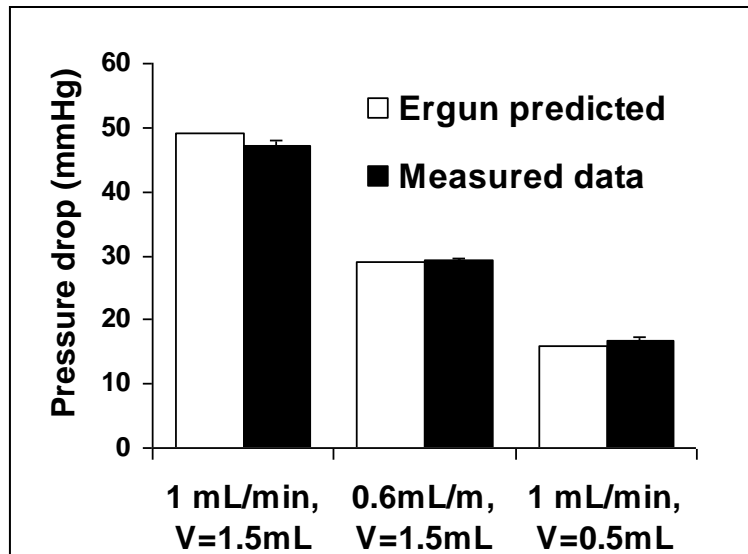


Fig. 2-17. Pressure drop across the column. Saline was pumped through the column with varying flow, pressure was measured at column inlet and outlet, and transcolum pressure was calculated. Data presented as mean \pm SEM. $n=6-11$, $p>0.4$ versus pressure predicted by the Ergun equation by t-test.

contamination was likely due to poor linkage of the hemoglobin to the beads. We eliminated this contamination by washing the beads extensively in 5 M guanidine before loading the plasma to remove any hemoglobin that was not tightly bound to the bead.

The purification of haptoglobin was scaled up to 100 mL beads/lot. There remained some apolipoprotein-A1 impurity within the haptoglobin elution, however the purity of haptoglobin across the entirety of the 3.5 M elution fractions is estimated at greater than 95%. A simple method to selectively remove apolipoprotein-A1 from the eluted fractions has subsequently been developed, wherein the fractions were concentrated using Amicon filters with a molecular weight cut-off of 50 kDa. The monomeric apolipoprotein-A1 (~25kD) passed through the pores (Fig. 2-8), while the multimeric haptoglobin was retained and concentrated.

A second means to remove apolipoprotein-A1 from the haptoglobin product using Glycine-HCl elution was attempted. This method did not separate apolipoprotein-A1 from haptoglobin in the fractions. Additionally, a large fraction of haptoglobin was eluted along with many nonspecific proteins (fractions #5-12; Fig. 2-9). To reduce the cost of haptoglobin purification, the hemoglobin-column was regenerated in 5M guanidine. The regeneration was successful for continued haptoglobin purification, however the yield steadily declined.

Early attempts to bind commercial haptoglobin to the beads were successful. With an initial binding solution concentration of 5 mg/mL haptoglobin, 98% of the haptoglobin was determined to have bound to the beads. The

method proved to be highly efficient to bind haptoglobin to the sepharose beads. It should be noted that the beads' capacity to bind protein is 25mg/mL but only 20% of that capacity was used. The optimum binding concentration of haptoglobin for this media with respect to the hemoglobin-binding capacity was determined when scale-up production of purified native haptoglobin was underway. The hemoglobin-binding capacity of haptoglobin-beads increased with increasing haptoglobin content until peaking at 15.9 mg haptoglobin/mL bead, therefore 15.9 mg haptoglobin/mL bead was determined to be the optimum concentration.

Sterilization and LPS removal from the haptoglobin-beads are necessary before they are introduced into the blood-contacting filter. The minimum ethanol requirements for sterilization have been determined. 50% ethanol was used because of the greater retention of activity. All three means to remove LPS or LPS activity reduced the level of activating LPS to below the significance threshold (1 ng/mL). The use of polymyxin and Pyroclean did not affect the hemoglobin-binding activity of the haptoglobin-beads. However, Triton X-114 reduced the hemoglobin-binding activity by 11% and did not readily wash out of the beads. Pyroclean was therefore used to remove LPS.

To explore the binding of hemoglobin to the haptoglobin beads in plasma, native plasma haptoglobin must be depleted. The spent plasma retained from haptoglobin purification was depleted of haptoglobin. There is an unknown protein of about 55 kD that crossreacts with the anti-haptoglobin antibody. This

protein does not bind hemoglobin as it is not present in the purified haptoglobin preparation (Fig. 2-14 lane 3).

We exposed the haptoglobin beads to haptoglobin-depleted plasma for one hour before performing a quantitative hemoglobin-binding assay. There was no indication of surface fouling or protease inactivation during 1 hour of incubation in plasma. The minimal amount of hemoglobin that was seen on the protein stained gel (Fig. 2-15b) was likely due to incomplete washing. Perhaps the beads were not washed long enough for the hemoglobin internalized within the porous structure of the beads to equilibrate with the wash solution.

Purified native haptoglobin was bound to the beads in a similar manner as described for commercial haptoglobin. The hemoglobin-binding activity of beads prepared with these two haptoglobin sources was compared. The purified native haptoglobin, when bound to beads, had a 60% greater hemoglobin-binding activity than that bought from Sigma. Beads were produced in large lots and used in successive experiments over a period of time. Loss of hemoglobin-binding capacity was quantified. Maintaining haptoglobin-beads at 4°C in 50% ethanol retains the hemoglobin-binding capacity for at least 11 weeks.

Prediction of transcolum pressure in a hemoperfusion circuit is important for setting operational parameters to within safety margins of the column (transcolum pressure <400 mmHg) and the passing blood cells (shear stress < 50 N/M², Appendix B)[15]. A screen-bounded column filled with soft beads has the potential for unexpected pressure increase and/or flow decrease due to beads plugging the open area of the screen and collapsed beads reducing the

effective void fraction. These may result in variable flow and poor pumping conditions. The pressure tests performed (Figure 2-17) indicated that transcolumn pressures were not significantly different than predicted by application of the Ergun equation (1) and unexpected pressure increase did not occur under the flow parameters tested. Additionally, flow was observed to be radially uniform and did not produce channeling within the column (Chapter 4, Figure 4-1B.)

Haptoglobin-bound beads that bind to hemoglobin have thus been developed. Additionally, a column to house the beads for filtration under flow have been developed and characterized. Lastly, a miniaturized circuit with the described bead-filled column inline has been developed for *in situ* filtration of rats.

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Chapter 3

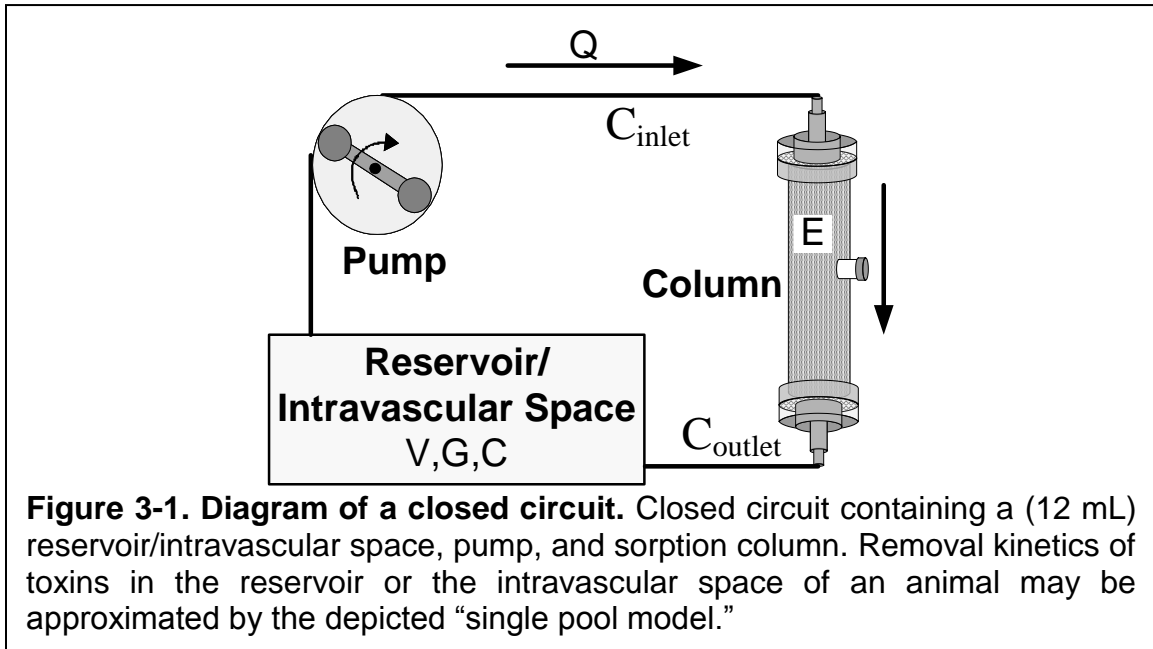
A model for predicting toxin removal during hemoperfusion

INTRODUCTION

Sepsis is a syndrome that is associated with multiple organ failure and significant morbidity and mortality. It affects 700,000 patients annually[1], is the 10th leading cause of death overall in the United States[2] and is rising in incidence[3]. The pathogenesis of sepsis is mediated by circulating toxins[4, 5]. There is broad interest in treating sepsis with hemoperfusion, an extracorporeal technology in which blood-borne toxins can be removed through direct contact with an adsorbent, often a sorption column[6, 7].

The application of such technology to *in vivo* systems can be a long and costly endeavor, thus it is important to utilize predictive models of toxin removal to first explore feasibility and then establish operating parameters. We have therefore established and tested a simple predictive model of toxin removal based on column properties that are easily determined experimentally. Hemoglobin was used for model development, as extracellular hemoglobin is an intravascular toxin associated with sepsis [Chapter 1] that can easily be measured spectrophotometrically[8]. Physiologic clearance of extracellular hemoglobin is mostly mediated by the tetrameric plasma protein haptoglobin, which binds quickly and irreversibly ($k \sim 6 \times 10^6 \text{ m}^{-1} \text{ sec}^{-1}$, binding affinity $K_d \sim 1 \text{ pmol/L}$)[9] to hemoglobin with 1:1 stoichiometry.[10, 11] We used haptoglobin-bound beads in sorption columns due to these favorable hemoglobin-binding properties.

Single pool model A simple model of extracellular hemoglobin removal



by an extracorporeal column in a closed circuit is the single pool model (Figure 3-1), where Q is flow (mL/min), and C is intravascular/reservoir hemoglobin concentration (mg/dL), where V is the volume of the reservoir or intravascular space (mL), G is generation rate of hemoglobin due to hemolysis (mg/min), and E is the column efficiency at hemoglobin removal (dimensionless). G is a first order irreversible reaction that is dependent on intracellular hemoglobin concentration and independent of extracellular hemoglobin concentration. This model also assumes that all the hemoglobin to be removed is in the intravascular compartment (a single-pool model is appropriate). If V is well mixed and V , Q , G and E are constant, then the plasma concentration of hemoglobin over time (t) can be expressed as the exponential decay:

$$C(t) = C_0 e^{-\frac{Q}{V}Et} + \frac{G}{QE} \left(1 - e^{-\frac{Q}{V}Et} \right). \quad (2)$$

However, as the filter capacity approaches saturation, E changes according to

$$E(t) = f \int_0^{t-dt} \frac{Q}{V} C(t) E(t) dt \quad (3)$$

an equation that must be determined empirically and solved for $E(t, C, Q, V)$, which describes E as a function of the column-bound hemoglobin. This may be accomplished by two distinct methods. In the first, the concentration of toxin within a closed circuit may be measured at various times and flows. A model of E can then be derived based on Equation 3 and generalized to predict toxin kinetics at other filtration conditions. In the second method, the saturation-dependency of E is quantified with single passes of constant concentration toxin in an open system. The relationship between efficiency and saturation (absorption isotherm) can then be used to model toxin concentration in a closed circuit. This paper explores the latter; applying empirically-derived absorption isotherms to model hemoglobin removal kinetics within a single-pool closed circuit *in vitro* and *in vivo*.

MATERIALS AND METHODS

Construction of sorption media

Haptoglobin (purified from human plasma as previously described in Chapter 2) was bound to N-hydroxysuccinimide (NHS)-Activated Sepharose Fast Flow 4 Beads (Amersham Bioscience; diameter range 45-165 μm , mean 90 μm). The beads were prepared as per manufacturer instructions: the beads reacted with a 10 mg/mL haptoglobin solution at 4°C overnight, and then unreacted binding sites were blocked with Tris-HCl (0.2M, pH 8; Fisher). All active sites on

the control-beads were blocked with Tris-HCl. The beads were sterilized in ethanol (50%, 24 hours) and stored at 4°C, conditions at which hemoglobin-binding activity was stable for at least 6 months.

Measurement of hemoglobin in solution

In vitro hemoglobin concentration was determined spectrophotometrically using the protocol of Harboe[8] which has recently been further validated by Fairbanks[12]. Briefly, absorbencies of saline-diluted samples were measured at 380, 415, and 450 nm (μ Quant adjustable wavelength spectrophotometer, Bio-Tek Instruments, Inc.) transformed by

$$C_{\text{Hemoglobin}} = 0.863[2A_{415} - (A_{380} + A_{450})], \quad (4)$$

and interpolated from a standard curve. The quantity of hemoglobin bound by the haptoglobin-beads was calculated from the difference in hemoglobin concentration between solutions filtered by haptoglobin-beads and control-beads, multiplied by the volume pumped (in open systems) or volume of the circuit (in closed systems). Haptoglobin-depleted plasma was used in all *in vitro* experiments as the spectrophotometric assay does not differentiate between hemoglobin and haptoglobin-hemoglobin complex, and circulating haptoglobin levels are depleted when hemolysis is clinically evident[13].

Determination of hemoglobin binding under flow (breakthrough)

Hemoglobin-binding properties of the haptoglobin-column were determined under flow conditions. Pooled heparinized human plasma was depleted of haptoglobin by passing through a column of hemoglobin-bound beads (Sigma). Hemoglobin solutions of various concentrations (50, 30 mg/dL in

haptoglobin-depleted plasma or saline) were pumped at various flows (0.6, 1 mL/min) through the column (1.5 mL of packed haptoglobin- or control-beads) and collected in 0.35 mL fractions (BioRad fraction collector model #2128) (Figure 3-2A). Hemoglobin binding to the control-column was found to be negligible. Hemoglobin bound to the haptoglobin-column was calculated from the concentration difference at the inlet and outlet of the haptoglobin-column and corrected by the concentration difference at the inlet and outlet of the control-column (Figure S3-1). The column volume and range of flow are appropriate for experimental rats (1.5mL haptoglobin-columns have a hemoglobin-binding capacity of 5.3mg, which corresponds to a maximum extracellular hemoglobin reduction of 47 mg/dL in a 300g rat. Continuous hemofiltration and hemodiafiltration are often administered at 2-4% of cardiac output to critically ill patients[14, 15], corresponding to a flow of 0.8 to 1.6 mL blood in a 300g rat) but can readily be scaled up[16].

Application of the Freundlich isotherm

The Freundlich isotherm is an empirical equation that models the equilibrium adsorption of an analyte in solution onto an adsorbent[17] (column) and is defined by:

$$\frac{q}{q_{\max}} = \left(\frac{C_{\text{outlet}}}{C_{\text{inlet}}} \right)^{\beta} , \quad \left(\frac{C_{\text{outlet}}}{C_{\text{inlet}}} \right) = \left(\frac{q}{q_{\max}} \right)^{\frac{1}{\beta}} \quad (5a,b)$$

where q = hemoglobin bound (mg)/ unit column (mg), q_{\max} = hemoglobin binding capacity (mg)/ unit column (mg), C_{outlet} and C_{inlet} = concentration of hemoglobin at

the column's outlet and inlet (mg/dL) respectively, and β is an empirically defined constant. The column saturation and the extent of hemoglobin "breakthrough" from the column are described by $\frac{q}{q_{\max}}$ and $\frac{C_{\text{outlet}}}{C_{\text{inlet}}}$, respectively. Freundlich isotherms were fitted to the measured breakthrough curve data using Equation 5a, the values for β determined by iteratively varying β to minimize the residual sum of squares between the isotherm and the data. An average β was then calculated from the aggregate data.

E was then expressly defined as a function of saturation. The amount of hemoglobin (mg) bound onto the column (per unit) over time is described by

$$q_t = \int_0^t C_i Q E_i dt, \quad (6)$$

where the hemoglobin binding efficiency of the column (E, dimensionless) is related to breakthrough and defined as

$$E_t = 1 - \left(\frac{C_{\text{outlet},t}}{C_{\text{inlet},t}} \right). \quad (7)$$

Substituting (5b) into Equation (7), the equation relating E_t as a function of saturation and q_t was derived:

$$E_t = 1 - \left(\frac{q_t}{q_{\max}} \right)^{\frac{1}{\beta}}. \quad (8)$$

A series of equations describing hemoglobin bound to the column and in solution was developed to model the decay of extracellular hemoglobin in a single-pool circuit (Figure 4.1). Saturation-dependent E values obtained from Equation (8)

were inputted into Equations (9) and (10) below. The hemoglobin bound to the column at time t is described as:

$$q_t = q_{t-\Delta t} + C_{t-\Delta t}QE_{t-\Delta t}\Delta t. \quad (9)$$

Time-dependent C was derived and expressed in Equation (10):

$$C_{inlet; t} = C_t = \frac{(C_{t-\Delta t}V - [q_t - q_{t-\Delta t}])}{V}. \quad (10)$$

To predict C_t *in vitro* and *in vivo*, Equations (8), (9), and (10) were solved simultaneously with increasing time ($\Delta t=0.1$ min) using relevant constants for $C_{t=0}$, Q, V, β , and q_{max} . When applying the model to closed circuits, the time domain was transformed into volumetric domain (volume pumped/volume of circuit, or number of complete circulations) to generalize the time-dependent model across flows and circuit volumes.

***In vitro* removal of hemoglobin from a closed circuit**

To explore the hemoglobin removal kinetics in a closed circuit analogue of a rat, hemoglobin solutions of 50 mg/dL in saline or haptoglobin-depleted plasma were recirculated (0.6 mL/min; Econo Gradient Pump, BioRad) through a circuit (3 mL saline priming volume) with an inline column (1.5 mL of haptoglobin- or control-beads) and a reservoir volume analogous to the nominal plasma volume of a 275g rat (12 mL), as depicted in Figure 4.1. The circuit was purged of air by circulating the priming solution until all the bubbles were removed. The reservoir was sampled periodically and hemoglobin concentration measured. It was assumed that mixing occurred only in the reservoir and that the hemoglobin-free saline prime diluted the reservoir concentration at a rate of $C_{initial}Q/V \text{ mg}^1\text{mL}^{-1}\text{min}^{-1}$, without any hemoglobin removal by the column, for the time duration of

V_{priming}/Q min. Differences between the predicted and the measured concentrations were analyzed for significance by repeated measures ANOVA. Precision of the model was assessed by varying β by 2 standard deviations from the mean (95% confidence interval of β).

***In situ* removal of extracellular hemoglobin from rats (hemoperfusion)**

Rats were anesthetized (isoflurane; 2.5%, 1.5% maintenance), cannulated (bilateral femoral artery and vein, 24Ga, BD Insite) and connected to a primed (saline) heparinized (15 U/mL; American Pharmaceutical Partners, Schaumburg, IL) circuit, previously described in detail[18], containing a 1.5 mL haptoglobin- or control-column inline. Blood was pumped at 1 ± 0.1 mL/min for 65 minutes. Hemoglobin (27.5 mg/kg; Sigma) was injected into the circuit post-column at $t = 5$ minutes and extracellular hemoglobin measured in blood sampled pre-column at $t = 4, 10, 25, 45,$ and 65 minutes. The hemoglobin-binding capacity of the haptoglobin-columns used to filter hemoglobin from rats (5.3 mg) was 26% greater than the hemoglobin-binding capacity of the columns used *ex vivo* (3-2 mg) due to batch differences of haptoglobin-beads. Extracellular hemoglobin was assayed spectrophotometrically using a modified Drabkin assay (Sigma, St Louis, MO)[19] and normalized to hematocrit, which accounted for dilution due to the circuit priming volume. Native circulating haptoglobin was measured from blood sampled prior to injection of hemoglobin ($t = 4$ min) by ELISA (Immunology Consultants Laboratory, Newberg, OR). The measured hemoglobin concentrations in blood treated with the haptoglobin-column were adjusted by adding the apparent reduction in hemoglobin from the blood treated with the

control-column, thus accounting for endogenous removal of extracellular hemoglobin by the rats. Native circulating haptoglobin interferes with removal of extracellular hemoglobin by the haptoglobin-column (unpublished data). All measured concentrations of hemoglobin in blood treated with the columns were adjusted by subtracting the concentrations of extracellular hemoglobin bound to circulating native haptoglobin according to Equations (11) and (12):

$$[\text{circulating hemoglobin}]_{\text{haptoglobin-normalized}} = [\text{circulating hemoglobin}]_{\text{measured}} - [\text{circulating hemoglobin}]_{\text{haptoglobin-bound}} \quad (11)$$

The stoichiometry of hemoglobin binding to haptoglobin is 1:1, hence plasma-bound hemoglobin was calculated as

$$[\text{circulating hemoglobin}]_{\text{haptoglobin-bound}} = [\text{circulating haptoglobin}]_{t=4\text{min}} \left(\frac{\text{molecular weights of hemoglobin}}{\text{haptoglobin}} \right) \quad (12)$$

Precision of the model was assessed by varying β by 2 standard deviations from the mean (95% confidence interval).

Table 3-1. Breakthrough at column saturation of 0.1, 0.5, 0.9. Data presented as mean \pm SD

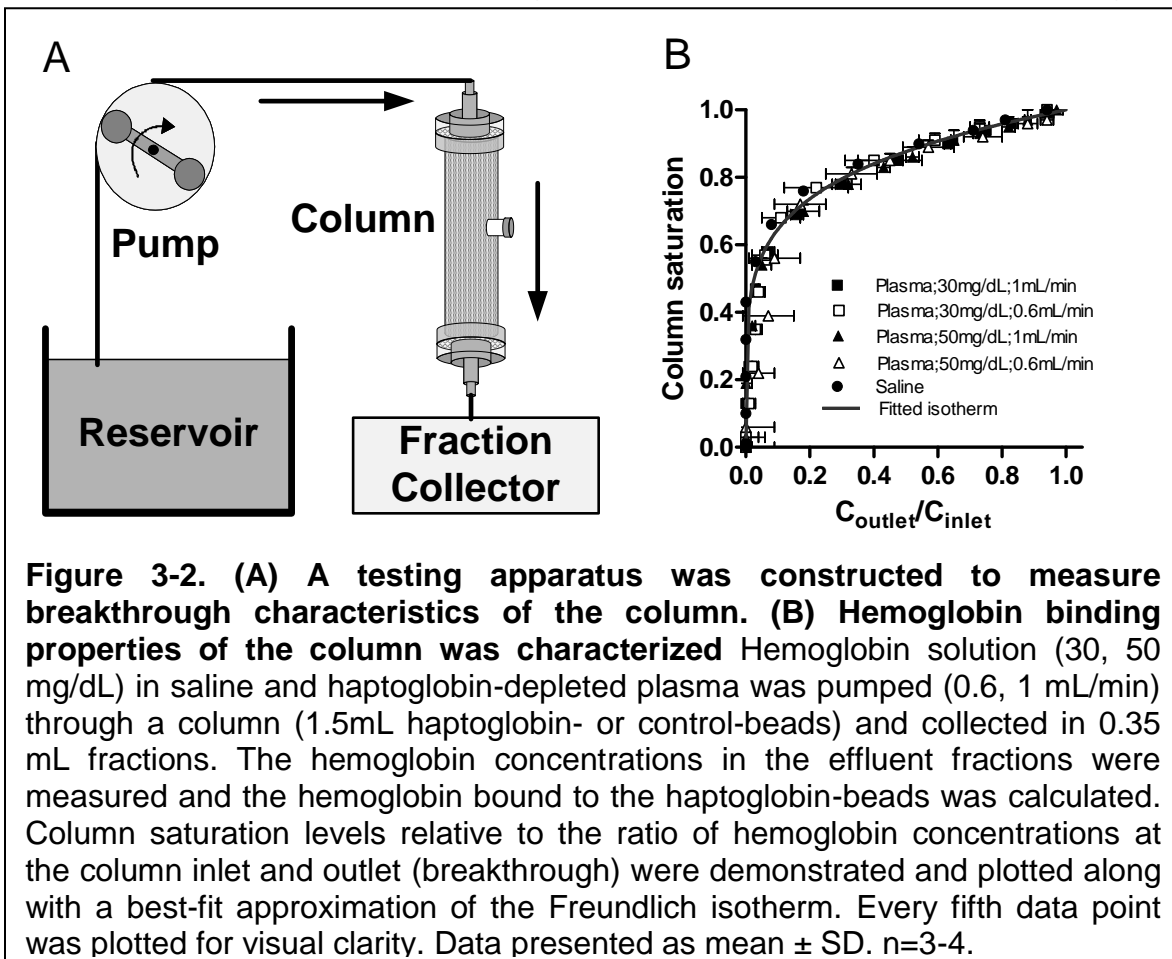
| Saturation | 0.1 | 0.5 | 0.9 |
|----------------------------|-----------------|-----------------|-----------------|
| Plasma;30mg/dL; 1mL/min | 0.02 \pm 0.02 | 0.04 \pm 0.01 | 0.60 \pm 0.02 |
| Plasma;30mg/dL; 0.6mL/min | 0.00 \pm 0.02 | 0.05 \pm 0.03 | 0.55 \pm 0.07 |
| Plasma; 50mg/dL; 1mL/min | 0.02 \pm 0.03 | 0.04 \pm 0.02 | 0.60 \pm 0.04 |
| Plasma;50mg/dL; 0.6mL/min | 0.02 \pm 0.07 | 0.08 \pm 0.08 | 0.64 \pm 0.07 |
| Saline; 30mg/dL; 0.6mL/min | 0.00 | 0.02 | 0.54 |
| Fitted isotherm | 0.00 \pm 0.00 | 0.03 \pm 0.01 | 0.57 \pm 0.03 |

RESULTS

Isotherm relationship is determined from the measurement of hemoglobin binding under flow

To characterize how the haptoglobin beads perform under flow conditions suitable for rat extracorporeal filtration, haptoglobin-depleted plasma and saline solutions of hemoglobin were pumped in a single pass through sorption columns of packed control- and haptoglobin-beads as depicted in Figure 3-2A, and the concentration of hemoglobin at the column outlet was measured (Figure 3-2B).

As tabulated in Table 1, column saturation as a function of breakthrough $\left(\frac{C_{outlet}}{C_{inlet}}\right)$ was independent of flow and hemoglobin concentration over the tested ranges.



Total hemoglobin-binding capacity of the columns reached $64\pm 3\%$ before the breakthrough exceeded 10%. The average best-fit Freundlich isotherm was calculated at $\beta = 0.19\pm 0.2$.

Model predicts *in vitro* hemoglobin removal from a closed circuit.

Hemoglobin solutions (50 mg/dL) in saline and haptoglobin-depleted plasma were filtered by haptoglobin- and control-columns in a closed circuit containing a 12 mL hemoglobin reservoir (Figure 3-3). Select reservoir hemoglobin concentrations are presented in Table 3-2. As depicted in Figure 3-3,

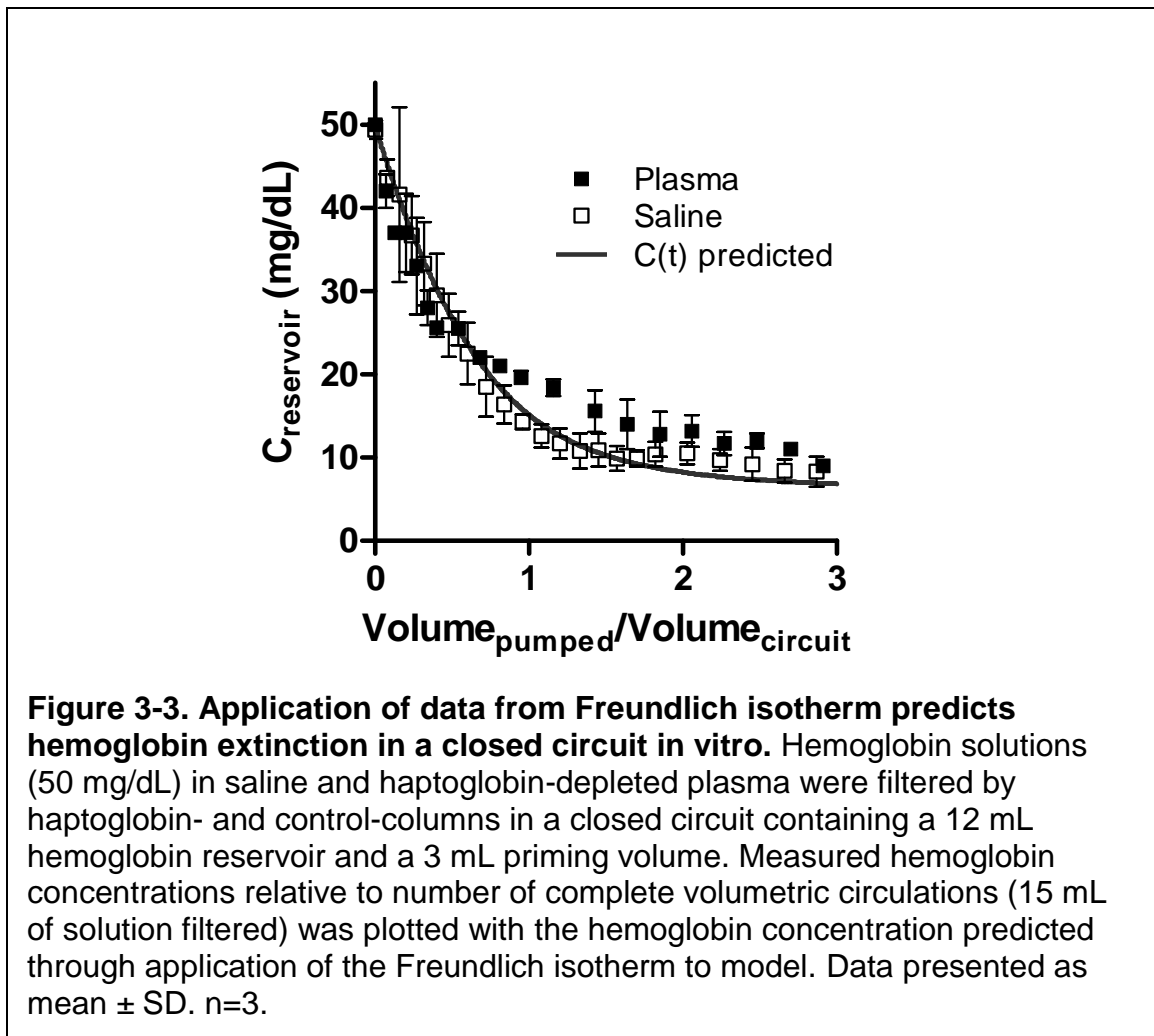


Figure 3-3. Application of data from Freundlich isotherm predicts hemoglobin extinction in a closed circuit *in vitro*. Hemoglobin solutions (50 mg/dL) in saline and haptoglobin-depleted plasma were filtered by haptoglobin- and control-columns in a closed circuit containing a 12 mL hemoglobin reservoir and a 3 mL priming volume. Measured hemoglobin concentrations relative to number of complete volumetric circulations (15 mL of solution filtered) was plotted with the hemoglobin concentration predicted through application of the Freundlich isotherm to model. Data presented as mean \pm SD. n=3.

the measured concentration of hemoglobin in the saline reservoir was accurately

predicted by the model. The measured concentration of hemoglobin in the plasma reservoir was accurately predicted for the first complete circulation of the circuit volume.

Although, the removal of hemoglobin from haptoglobin-depleted plasma was overestimated thereafter, the deviation was not significant ($p=0.78$ by RM ANOVA). The predicted concentration of hemoglobin at the 95% confidence limits of β deviated by an average ± 0.3 mg/dL with a maximum deviation of 0.5 mg/dL.

Table 3-2. Reservoir hemoglobin concentrations. Data presented as mean \pm SD.

| Volume_{pumped}/Volume_{circuit} (Circulation #) | 0.5 | 1.5 | 2.5 |
|---|----------------|----------------|----------------|
| Saline; 50mg/dL | 25.9 \pm 3.8 | 11.7 \pm 2.1 | 10.4 \pm 1.5 |
| Plasma; 50mg/dL | 25.5 \pm 2.0 | 14.0 \pm 3.0 | 12.0 \pm 0.9 |
| Model predicted C(t) | 26.7 \pm 0.0 | 10.3 \pm 0.3 | 7.3 \pm 0.2 |

Model predicts *in situ* hemoglobin removal from rats

Blood of rats with elevated exogenous hemoglobin was treated in situ with extracorporeal hemoperfusion. The haptoglobin-column removed 29 ± 12 mg/dL

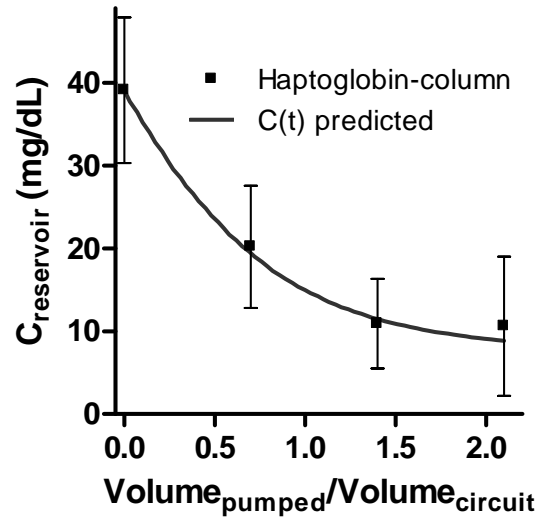


Figure 3-4. Application of data from Freundlich isotherm predicts hemoglobin extinction in vivo. Rats were connected to extracorporeal circuits containing haptoglobin- and control-columns inline and hemoglobin was injected (27.5mg/kg) into the circuits post-column. Blood was filtered in situ for 60 minutes and extracellular hemoglobin concentration was measured from precolumn samples. Measured hemoglobin levels in the rats filtered with haptoglobin-columns were adjusted using the measured hemoglobin levels in the rats filtered with control-columns. Hemoglobin levels were then normalized to native circulating haptoglobin and plotted, along with the hemoglobin levels predicted by application of the Freundlich isotherm, against the number of complete volumetric circulations. Data presented as mean \pm SD. n=7-8.

extracellular hemoglobin over two complete volumetric circulations (Figure 3-4).

The concentration of circulating haptoglobin at onset of filtration was measured (26 ± 4 mg/dL, n=15) and extracellular hemoglobin measurements were normalized for individual circulating haptoglobin concentrations. Application of the predictive model accurately predicted the haptoglobin-normalized data (Table 3, Figure 3-4). The predicted concentration of hemoglobin at the 95% confidence

limits of β deviated by an average of ± 0.3 mg/dL with a maximum deviation of 0.5 mg/dL.

Table 3-3. Extracellular hemoglobin concentrations. Data presented as mean \pm SD.

| Volume_{pumped}/Volume_{circuit} (Circulation #) | 0 | 0.7 | 1.4 | 2.0 |
|---|----------------|----------------|----------------|----------------|
| Haptoglobin-column | 39.1 \pm 8.8 | 20.2 \pm 7.4 | 10.9 \pm 5.4 | 10.6 \pm 8.4 |
| Model predicted C(t) | 39.1 \pm 0.0 | 19.8 \pm 0.1 | 11.9 \pm 0.5 | 9.5 \pm 0.5 |

DISCUSSION

There are two distinct strategies to model toxin removal in a closed system by an adsorptive column. The first is to deduce column behavior through empirical observation of system behavior and the second is to deduce system behavior through empirical observation of column behavior. Others have described a method to use system behavior to model transport characteristics of the column.[20] In contrast, this paper discusses measuring transport characteristics of the column that are then used to model system behavior.

There are advantages to modeling system behavior from empirically determined column characteristics. It is difficult to generalize an empirically-based model of a transitory system (closed circuit) as the column E , C_{inlet} , and C_{outlet} are constantly changing. Additionally, variability in reservoir mixing, sampling volume, limited sampling number, and priming volume can accumulate to significant error. Breakthrough curves in a single-pass system (Figure 3-2A) provided data to generalize column saturation-dependant relationships of C_{outlet} to C_{inlet} (Figure 3-2C). The data measured from the single-pass system contained far less inherent error than the transitory system.

Important working parameters can be determined by this data, such as a range of operating blood flows, hemoglobin $C_{initials}$, and column hemoglobin-binding capacities. Figure 3-2C presents the breakthrough data as a function of column saturation, which tends to normalize the flow and C_{inlet} effects on breakthrough. It is nonetheless significant to observe flow- and inlet concentration- independence of the column saturation relative to hemoglobin

breakthrough over the ranges tested. Further studies can identify the flow threshold above the tested range where the saturation-dependent breakthrough deviates due to insufficient residence time of the hemoglobin. Figure 3-2C also indicates no significant difference in breakthrough when removing hemoglobin from saline and haptoglobin-depleted plasma, indicating that haptoglobin-depleted plasma did not significantly inhibit the binding of hemoglobin to the column. The effectiveness of a column to remove an analyte can be gauged by its efficiency relative to its saturation. For the column to remove hemoglobin from solution with less than 10% breakthrough (>90% efficiency), the hemoglobin capacity of the column must exceed the hemoglobin to be removed by 1.6 fold (100/64%). Should the column surpass 64% saturation, the rate of removal will be suboptimal.

The Freundlich isotherm as described in Equation 5a[16] is determined based on the measured data plotted in Figure 3-2C. The convex shape of the isotherm categorizes the absorption of hemoglobin as favorable[16], implying binding of hemoglobin to the column even at low concentration. The observed saturation-breakthrough relationship in Figure 3-2C was then used to model hemoglobin removal from a closed circuit (Figure 4.1). Hemoglobin in saline and haptoglobin-depleted plasma was filtered for three complete circulations of volume. As depicted in Figure 3-3, the reservoir hemoglobin was reduced over the course of the filtration in a manner predicted through application of the empirically-derived Freundlich isotherm to the model (Equations (8), (9), and (10)). The model more accurately predicted the removal of hemoglobin from

saline than from plasma, perhaps due to physical or thermodynamic interference in the adsorption of hemoglobin on the mostly saturated column by plasma molecules at lower hemoglobin concentrations; however, the differences between the empirical and predicted data were not significant.

The columns were used to filter exogenous hemoglobin from live rats *in situ*. Figure 3-4 depicts the extracellular hemoglobin concentration versus volume pumped/volume_{circuit} after adjusting for hemoglobin removal from circulation by the rat through various endogenous mechanisms (comparable to the published acute endogenous clearance of extracellular hemoglobin in man^[21] and rat^[22] of approximately 12 mg/dL/hr). After taking native haptoglobin into account as well, the model accurately predicted the removal of extracellular hemoglobin, unbound to circulating haptoglobin, by the control-normalized haptoglobin-columns (Figure 3-4). Taken together, a predictive model of hemoglobin concentration based on empirically determined saturation-dependant breakthrough (and therefore column efficiency) was able to accurately predict hemoglobin removal from saline and haptoglobin-depleted plasma *in vitro* and *in vivo*.

While the described technology is enabling for the removal of hemoglobin, the platform can be configured for use against other toxins. New configurations should be assessed by breakthrough curve analysis to predict operational parameters. The more that is known about endogenous interactions with the toxin, the more accurate the predictions will be.

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Supplementary data

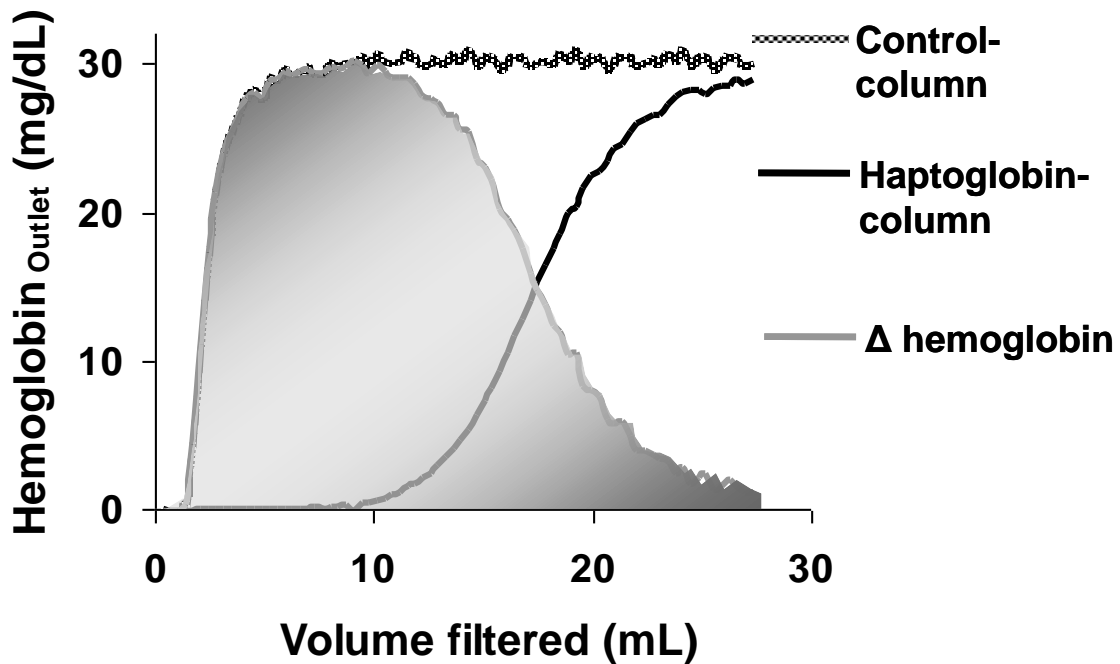


Figure S3-1. Hemoglobin binding properties of the column was characterized Representative profile of hemoglobin concentrations at the column outlet as a function of filtered volume. The quantity of bound hemoglobin was calculated from the concentration difference (Δ hemoglobin) at the inlet and outlet (effluent fractions) of the haptoglobin-column and corrected by the transcolumn concentration difference of the control-column.

Chapter 4

**Extracorporeal removal of plasma hemoglobin during sepsis
decreases cytokine levels and attenuates organ damage**

ABSTRACT

Sepsis, the most common cause of death in hospital intensive care units, is a syndrome mediated by cytokines and other toxins that cause organ damage. Hemoglobin is toxic when released extracellularly into plasma. Plasma hemoglobin levels, which are elevated during sepsis, significantly increase cytokine production by activated macrophages. Elevated hemoglobin dose-dependently increases lethality in septic mice (0 mg/kg= 0%; n=10, 20 mg/kg=26%; n=11, 200 mg/kg=70%; n=10, 2000 mg/kg= 100%; n=4). Here we describe a bead (sorption) column built by coupling haptoglobin to a solid phase matrix. Extracorporeal filtration using this column for one hour at 1mL/min significantly reduces plasma hemoglobin in rats ($64\pm 7\%$; n=7; $p<0.005$). During sepsis, in a standardized model of cecal ligation and puncture, the extracorporeal circuit reduces plasma levels of ICAM-1, IL-1 β , HMGB1, LDH, and AST. Thus, the haptoglobin-column can specifically eliminate hemoglobin from rats and reduce levels of proinflammatory cytokines and cellular damage in sepsis with hemoglobinemia.

INTRODUCTION

Severe sepsis, which has an overall mortality rate near 30%, is the most common cause of death in hospital intensive care units, and has an annual U.S. national cost of \$16.7 billion [1]. Research into this problem has created a now large scientific field focused on defining the biology and pathophysiology of inflammatory mediators in the development of human sepsis. It has become clear that cytokines produced during the “normal” course of infection can directly injure the host [2-4]. During sepsis, red blood cells become rigid and susceptible to hemolysis, generating an increased concentration of extracellular hemoglobin [5]. Plasma hemoglobin is toxic in part because it alters the expression of redox-sensitive genes and increases the release of release of cytokines [6-8]. Hemoglobin and bacterial lipopolysaccharide (LPS) synergistically increase release of TNF from activated macrophages [9, 10]. Administration of hemoglobin during endotoxemia significantly increases mortality and cytokine production [11, 12]. We reasoned that the removal of plasma hemoglobin during sepsis would decrease the release of cytokines and protect organs from damage. Plasma hemoglobin clearance is normally mediated by haptoglobin, a tetrameric plasma glycoprotein that binds to hemoglobin with 1:1 stoichiometry [13, 14]. Haptoglobin-hemoglobin complexes are cleared and degraded by CD163+ tissue macrophages residing mostly in the liver and spleen [14, 15]. Circulating haptoglobin can become depleted in conditions of sepsis-related hemolysis, resulting in sustained elevations of plasma hemoglobin levels [16]. Here we develop an extracorporeal sorption column composed of haptoglobin-linked

beads that specifically remove plasma hemoglobin from circulation. Application of the column to hemoglobin solutions significantly reduces cytokine production by macrophages and protects tissue from injury in mouse sepsis.

MATERIALS AND METHODS

Hemoglobin-binding capacity and kinetics

Haptoglobin-beads and control-beads (100 μ L, 50% suspension) were incubated with hemoglobin (purified from rat erythrocytes; 150 μ L, 200 mg/dL saline). At 10, 30, and 45 seconds, and 1, 5, 20, and 60 minutes, the suspensions were centrifuged and the supernatants were assessed for hemoglobin concentration as previously described in Chapter 3. The quantity of hemoglobin bound by the haptoglobin-beads was calculated from the difference in supernatant hemoglobin concentration between haptoglobin-beads and control-beads.

Column preparation

Column housings were assembled as described in Chapter 2. Briefly, the housings consist of Luer-containing perfusion connectors ($\frac{1}{4}$ " ID) fitted with a stopcock and sealed (UV cured polyurethane) at each end with heparinized screen (polyester, 37 μ m mesh, Cobe Laboratories, Denver, CO) to retain the beads. The inlet and outlet were manufactured by using increasing ID PVC tubing nested one inside another. Sterilized beads were introduced into the housing through the stopcock (Fig. 4-1B).

Hemoglobin binding under flow (breakthrough)

Hemoglobin (purified from rat erythrocytes) solutions of various concentrations (50, 30 mg/dL in haptoglobin-depleted plasma or saline) were pumped (Econo Gradient Pump, BioRad) at various flows (0.6, 1 mL/min) through the column (1.5 mL of packed haptoglobin- or control-beads) and

collected in 0.35 mL fractions (BioRad fraction collector model #2128). Column-bound hemoglobin was calculated from the concentration difference at the inlet and outlet of the haptoglobin-column and corrected by the concentration difference at the inlet and outlet of the control-column.

Filtration of hemoglobin solutions (for cell culture and animal work)

Hemoglobin solution (human methemoglobin, Sigma; 2000 mg/dL normal saline) was single-pass filtered through scaled-up sorption columns (haptoglobin-column or control-column) that were sterilized with ethanol and stripped of endotoxin (PyroCLEAN buffer, Alerchek, Portland, MA). Unbound hemoglobin in the column was chased with one column volume of saline. The effluent was concentrated above Amicon filters (Millipore; 10 kD molecular weight cutoff) to the starting volume of the hemoglobin solution and the hemoglobin concentration was measured. The hemoglobin solutions were then filter sterilized (Uniflo-25 0.45 μm , Schleicher & Shuell) and stored at -20°C .

Animals

Adult male BALB/c mice 8 to 12 weeks-old (20-25g; Taconic) and adult male Sprague Dawley rats 8 to 16 weeks-old (250-350g, Taconic) were housed at 25°C on a 12-hour light/dark cycle, and acclimatized for one week before experiments were conducted. Water and regular rodent chow were available *ad libitum*. Experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, North Shore-LIJ Health System.

Cecal Ligation and Puncture (CLP) with Elevated Plasma Hemoglobin in Mice

To determine effects of increased plasma hemoglobin in polymicrobial sepsis, mice were first subjected to the CLP procedure and then injected with hemoglobin. After anesthesia with an intramuscular injection of ketamine (100 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (8 mg/kg; Boehringer Ingelheim), a 1-2cm midline abdominal incision was made to expose the cecum. After ligation 1 cm from the tip, the cecum was punctured once with a 22-gauge needle, and small amount of stool was extruded (1mm). The cecum was placed back into its normal intraabdominal position, and the wound was closed with surgical staples. All animals received saline (0.9% s.c., 20 ml/kg of body weight) resuscitation, and a single dose of antibiotic (0.5 mg/kg of imipenem s.c.; Primaxin, Merck). Sham CLP was performed as above, except the cecum was returned to the peritoneum without ligation or puncture. Immediately following sham or CLP, hemoglobin solutions of various concentrations (0, 20, 200, 2000 mg/kg; 0.3 mL) were injected (29G, I.V.; Becton Dickinson). In other experiments, filtered and control-filtered hemoglobin solutions (prefiltered hemoglobin concentration equivalent to 200 mg/kg; 0.3 mL) were injected directly into the spleen before the peritoneal cavity was closed. Mice were followed for 21 days for survival. In parallel experiments, mice were euthanized 24 hours post-CLP and their blood collected.

Cecal Ligation and Puncture in Rats

After anesthesia (isoflurane, 2.5%), a 2 cm midline abdominal incision was made to expose the cecum. After ligation 2 cm proximal to the tip, the cecum was punctured twice with a 22-gauge needle, and small amount of stool was extruded (3 mm). The cecum was returned to the abdominal cavity, and the abdominal wall was closed in one layer with running 4-0 prolene suture. All animals received saline resuscitation (0.9% s.c., 30 ml/kg of body weight), and a single dose of antibiotic (3 mg/kg of imipenem s.c.; Primaxin, Merck). Blood was sampled via retroorbital sinus into heparinized tubes. Control samples (Day 0) were obtained prior to CLP.

Cell Culture

Murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Rockville, MD), were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini, Calabasas, CA), penicillin (50 units/mL) and streptomycin (50 µg/mL; Life Technologies) in a humidified incubator under 5% CO₂. At 90% confluence, cells were mechanically removed and plated into 96-well microtiter plates at 2x10⁵ cells/well overnight. Primary human peripheral blood monocytes were isolated from leukocyte-rich human blood fractions (Long Island Blood Service, Melville, NY) and differentiated into macrophages as previously described [10]. Cells were washed and resuspended in serum reduced Opti-MEM I medium (Invitrogen, Carlsbad, CA) containing lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; 1ng/mL) and hemoglobin solution (control- or haptoglobin-filtered at various dilution) or saline was added (1:20 V/V).

Selective filtering of plasma hemoglobin from rat

Rats were anesthetized (isoflurane; 2.5%, 1.5% maintenance), cannulated (bilateral femoral vessels, 24Ga, BD Insyte) and connected to a primed (15 U/mL heparinized saline; American Pharmaceutical Partners, Schaumburg, IL) extracorporeal circuit (4.5mL prime) containing a haptoglobin- or control-column inline. Blood was pumped arteriovenously at 1 ± 0.1 mL/min for 65 minutes. Hemoglobin (27.5 mg/kg; Sigma) was injected over a one minute period into the circuit post-column at $t=5$ minutes and plasma hemoglobin measured from blood sampled pre-column at $t=4, 10, 25, 45,$ and 65 minutes. The sampling volume was replaced by normal saline. The cannulae were removed, the vessels were ligated, and the incisions closed with running suture. Sepsis was then induced by CLP as described above. Hemoglobin in plasma samples was quantified by modified Drabkin protocol [17]. Briefly, thawed plasma samples were diluted five times in Drabkin's Reagent (Ricca, Fufts IL) and incubated for 30 minutes. The absorption at 540 nm was quantified and the concentration determined by interpolating from a standard curve. Plasma hemoglobin levels were normalized to the hematocrit of the sample to account for hemodilution.

Measurements of cytokines and plasma enzymes

Cytokines (TNF, IL6) were measured by ELISA kits (R&D Systems) or quantitative array (Raybiotech, Norcross GA). HMGB1 was measured by western immunoblotting as previously described [18]. Plasma enzymes lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured by AniLytics, Inc. (Gaithersburg, MD).

Statistical analysis

Data in figures are expressed as means \pm SEM or SD as labeled. Unpaired two-tailed t-test was used to compare mean values between groups; p values < 0.05 were considered significant. Survival plots were calculated using the nonparametric Kaplan-Meier technique and differences between groups assessed by log-rank; p values < 0.05 were considered significant.

RESULTS

Haptoglobin-beads remove hemoglobin from solution

Haptoglobin bound to sepharose beads removes hemoglobin from solution, with over 70% of the equilibrium solid state hemoglobin binding within one minute (Fig. 4-1A). By 20 minutes, the haptoglobin-beads are fully saturated, removing 3.3 ± 0.14 mg hemoglobin per mL of beads from solution. Haptoglobin-bead performance was characterized under flow conditions suitable for rodent extracorporeal filtration by pumping haptoglobin-depleted plasma and saline solutions of hemoglobin through sorption columns of packed control- and haptoglobin-beads and measuring the concentration of hemoglobin at the column outlet. The haptoglobin-column, which is white at the onset, turns red as it binds hemoglobin. The reddening occurs in a radially symmetric “moving wave” pattern indicating that the hemoglobin binding to the packed haptoglobin-beads is homogeneous (Fig. 4-1B). The column efficiency, defined as $[\text{inlet concentration} - \text{outlet concentration}] / \text{inlet concentration}$ ($[(C_o - C_i)/C_i]$), as a function time is shown in Fig. 4-1C. The column maintains efficiency greater than 90% until at least 64% of its total capacity is depleted, independent of the hemoglobin concentration (Fig. 4-1D) and the flow conditions (Supplementary Figure 4-S1B). There is no significant difference in the column’s hemoglobin-binding capacity or in hemoglobin removal kinetics from saline and haptoglobin-depleted plasma (Fig. 4-1E). Additional data points are shown in supplementary Figure 4-S1B.

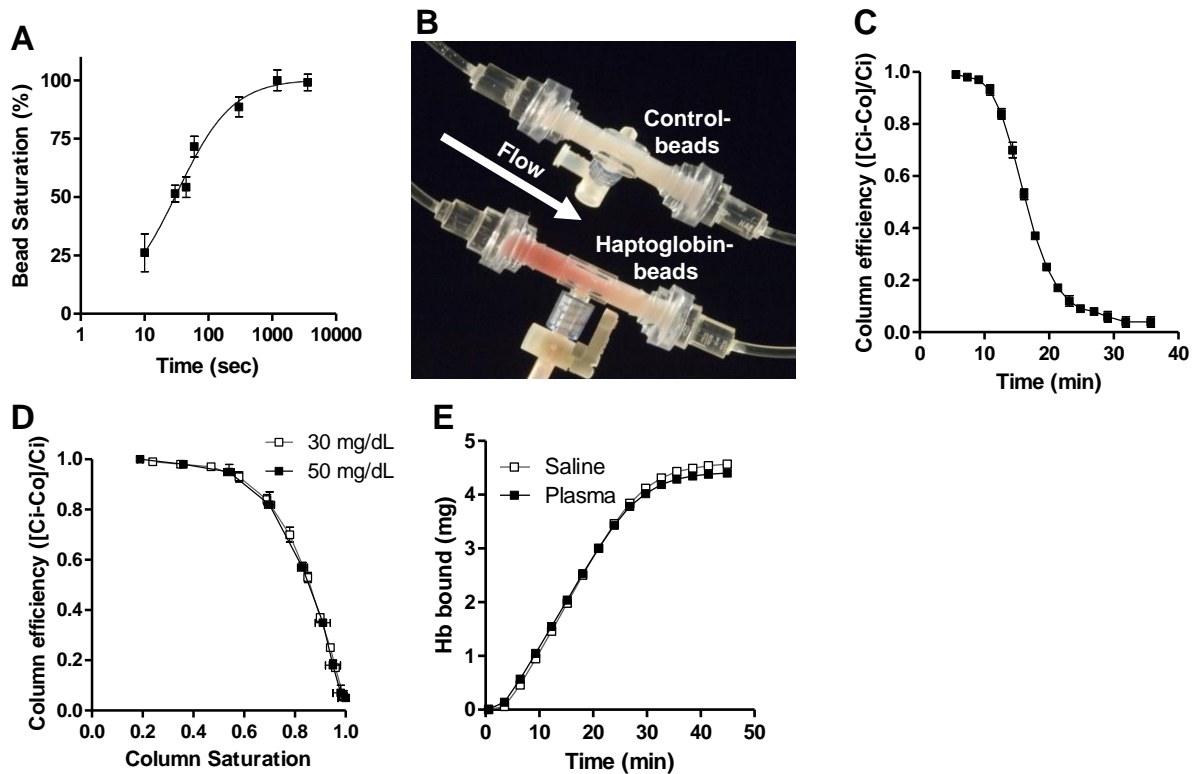


Figure 4-1. Characterization of hemoglobin uptake by haptoglobin beads in (A) batch and flow-through conditions; uptake efficiency is independent of (C) flow, (D) concentration, or (E) solvent. (A) Control- and haptoglobin-beads (100uL) were exposed to hemoglobin (150uL; 200mg/dL) in a batch reactor and centrifuged at various times. Hemoglobin concentration in the supernatant was measured spectrophotometrically. Hemoglobin bound to haptoglobin beads was calculated from the difference in supernatant hemoglobin concentration between control- and haptoglobin-beads. Data presented as mean \pm SD. n=3 experiments. Hemoglobin solutions of (B,D) 50 and (C,D,E) 30 mg/dL in (C-E) haptoglobin-depleted plasma and (B,E) saline were pumped at (B,C,D) 1 and (E) 0.6 mL/min through the column (1.5 mL of haptoglobin-bead or control-beads) and collected in 0.35 mL fractions. The hemoglobin concentration in the effluent fractions was determined. The quantity of bound hemoglobin was calculated from the concentration difference at the inlet and outlet (effluent fractions) of the haptoglobin-column and corrected by the concentration difference at the inlet and outlet of the control-column. **(B) Photograph of control- and haptoglobin-bead columns** at 10 minutes of single-pass filtration of hemoglobin in saline depicts characteristic hemoglobin uptake onto haptoglobin beads. Column efficiency ($[\text{inlet concentration} - \text{outlet concentration}] / \text{inlet concentration}$; $(C_o - C_i)/C_i$) is demonstrated as a function of (C) time and (D) column saturation. (E) The kinetic equivalence of hemoglobin uptake from haptoglobin-depleted plasma and saline are shown. (C-E) Data presented as mean \pm SD. n=4 experiments.

Removal of exogenous hemoglobin increases survival and decreases markers of organ damage.

Elevated plasma hemoglobin levels significantly enhance lethality in

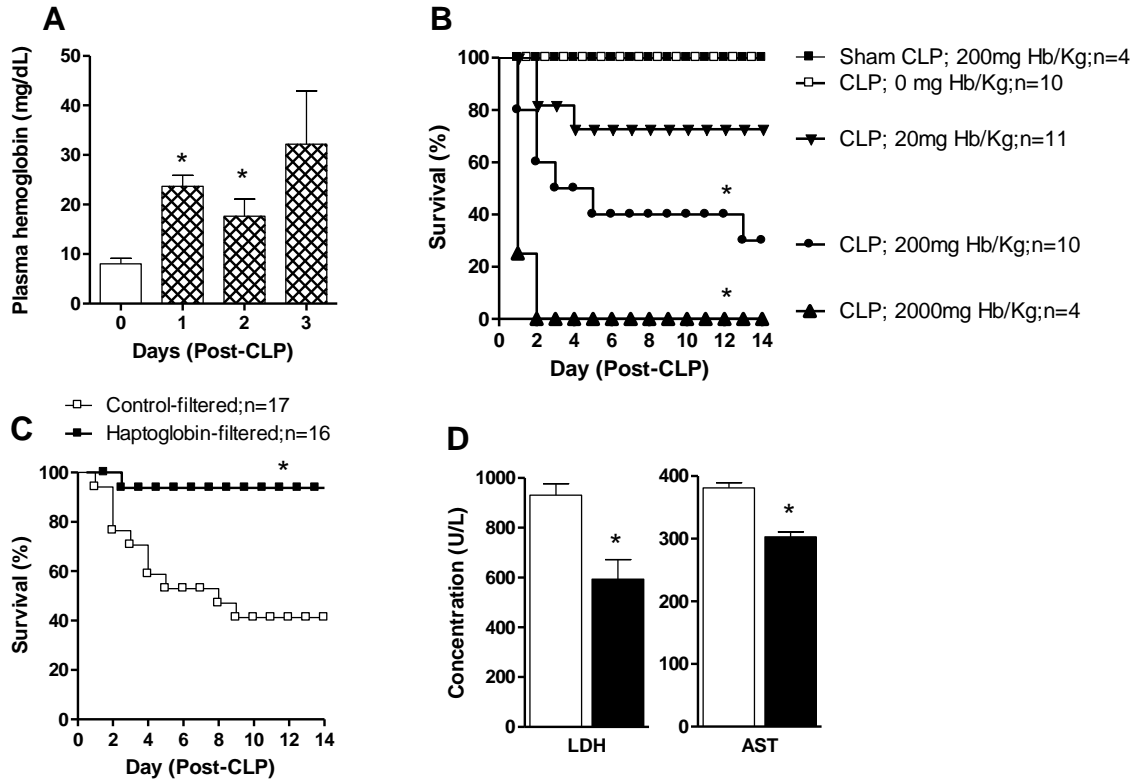


Figure 4-2. (A) Plasma hemoglobin is elevated after cecal ligation and puncture in rats. (B) Plasma hemoglobin increases lethality in septic mice. Filtration of hemoglobin solution decreases (C) lethality and (D) organ damage in septic mice. (A) Cecal ligation and puncture was performed on Sprague Dawley rats. Plasma was sampled at 24, 48, and 72 hours after puncture and plasma hemoglobin measured spectrophotometrically. *, $P < 0.05$ versus Day 0 (naïve); Day 0, $n = 8$; Day 1, $n = 45$; Day 2, $n = 22$; Day 3, $n = 6$. Data presented as mean \pm SEM. BALB/c mice were subjected to cecal ligation and puncture or sham surgery and hemoglobin (0.3 mL) injected (B) intravenous or (C,D) filtered or control-filtered hemoglobin (0.3 mL) injected into the spleen over 60 seconds. Data are shown as percent of animals surviving. (B) *, $P < 0.05$ versus 0 mg/kg hemoglobin by log-rank; $n = 4-11$. (C) *, $P < 0.05$ versus control-filtered hemoglobin by log-rank; $n = 16-17$. (B,C) Mice were followed for three weeks and no deaths occur after day 13. (D) A subset of mice were killed 24h after CLP surgery. Plasma levels of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured. *, $P < 0.05$ versus control-filtered; $n = 5$. Data presented as mean \pm SEM.

endotoxemia [11, 12]. Plasma hemoglobin levels are significantly elevated at day 1 and day 2 after the onset of sepsis induced by cecal perforation as compared to baseline levels at day 0 (Day 1=24±2 mg/dL; n=45, Day 2=18±3 mg/dL; n=22 vs. Day 0=8±1 mg/dL; n=8; p<0.05) (Fig. 4-2A). Administration of hemoglobin into BALB/c mice following cecal ligation and puncture (CLP) significantly increases lethality (Fig. 4-2B). Lethality increases with increasing hemoglobin dose (0 mg/kg= 0%; n=10, 20 mg/kg=26%; n=11, 200 mg/kg=70%; n=10, 2000 mg/kg= 100%; n=4). Filtration of hemoglobin solutions over the haptoglobin-bead column significantly reduces the toxicity of the solution when administered to septic mice (haptoglobin-column=6% mortality; n=16 vs. control-column= 59% mortality; n=17; p=0.002 by log-rank) (Fig. 4-2C). In this same group of mice, plasma markers of organ damage are significantly reduced at 24h following cecal perforation in the animals that receive hemoglobin solution subjected to haptoglobin-column filtration as compared to the control-column filtration (LDH= 594±78 U/L vs. 931±46 U/L; AST= 303±8 U/L vs. 381±9 U/L; n=5; p<0.05) (Fig. 4-2D).

Enhanced release of cytokines from hematopoietic cells has been implicated in the severity and lethality of sepsis [19, 20]. TNF, IL-6, and IL-1 α release from endotoxin-stimulated (1 ng/mL) murine RAW 264.7 cells and human primary macrophages increase by two logs when exposed to control-column filtered hemoglobin as compared to saline (Fig. 4-3A-D). Significant elevations in IL-1 β , IL-2, IL-10, M-CSF, and CCL5 (6±1 to 74±5 fold increase vs. saline, p<0.02) are also observed (Fig. 4-3B). The stimulatory effect on TNF and IL-6 is

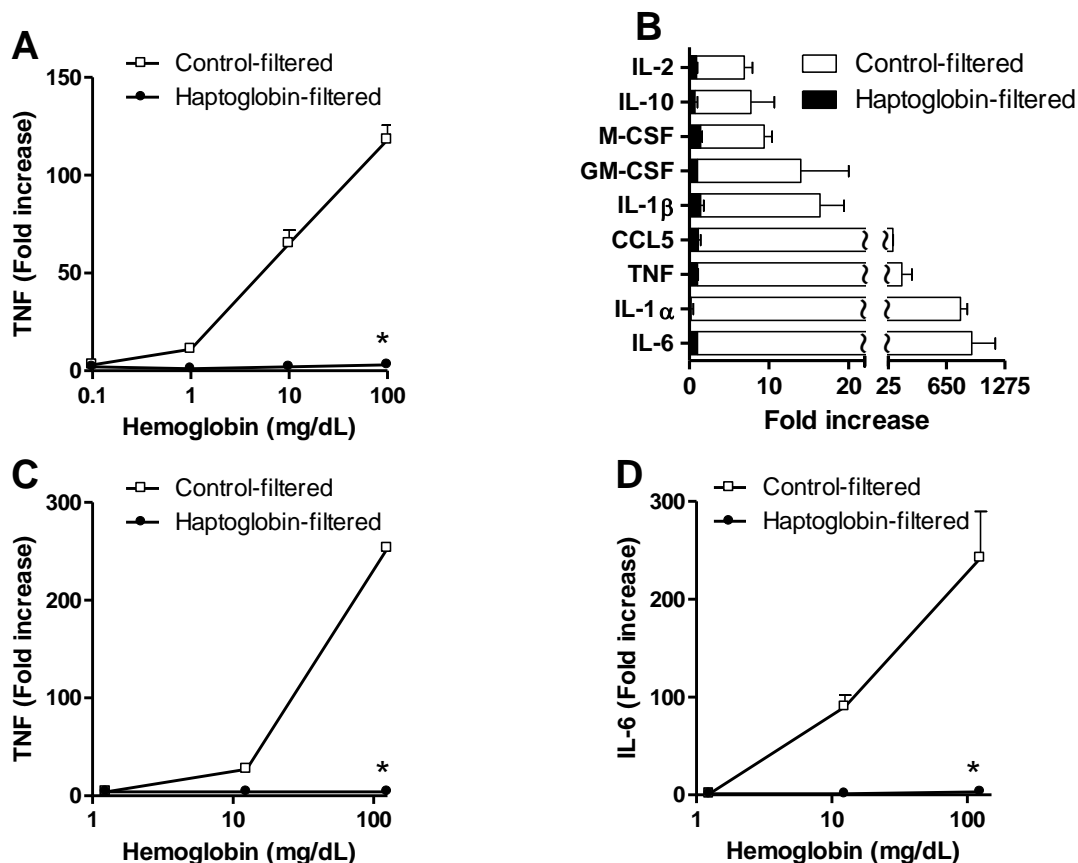


Figure 4-3. Filtration of hemoglobin abrogates inflammatory response *in vitro* in (A,B) murine and (C,D) human macrophages. A saline-hemoglobin solution (2000 mg/dL) was filtered in a single pass through control- or haptoglobin-column and added, along with endotoxin (1 ng/mL), to (A,B) murine macrophage-like RAW 264.7 cells or to (C,D) human primary macrophage at doses indicated for 12h. Levels of indicated cytokines were measured from the conditioned media. Murine and human TNF and human IL-6 were measured by ELISA. Murine GM-CSF, IL-1 α , IL-6, IL-1 β , IL-2, IL-10, M-CSF, and CCL5 were measured by quantitative cytokine array. Data are expressed as fold-stimulation by endotoxin with saline alone. *P<0.05 versus control-filtered; n=3-6. Data presented as mean \pm SEM.

hemoglobin-dose dependent (Fig. 4-3A,C), and removal of the hemoglobin reduces the cytokine and chemokine concentrations to control levels (Fig. 4-3A-D).

Extracorporeal filtering of blood in rat reduces plasma hemoglobin and reduces plasma cytokine and organ damage marker levels in sepsis

Rats with elevated plasma hemoglobin (27.5 mg/kg) subjected to extracorporeal blood filtration with haptoglobin-beads have significantly lower levels of plasma hemoglobin than rats filtered with control-beads (haptoglobin-beads=36±7%; n=7 vs. control-beads= 89±16%; n=8; p=0.005) (Fig. 4-4A). The reduction is evident by 20 minutes (p<0.04) and remains significant (p<0.01) throughout the hour-long procedure. The haptoglobin-beads abrogate the proinflammatory response relative to the control-column, indicated by a reduction in IL-1 β (5 fold), HMGB1 (5 fold), and ICAM-1 (2 fold) at 24h after the onset of sepsis. Furthermore, treatment with the haptoglobin-beads results in a 2-fold increase in the anti-inflammatory cytokine IL-10 over treatment with the control-column (Fig. 4-4B). Haptoglobin-column filtration significantly reduces plasma LDH and AST as compared to control-column filtration (LDH= 376±99 U/L vs. 1339±180 U/L; AST= 222±95 U/L vs. 647±39 U/L; n=3; p<0.03), indicating that removing hemoglobin with the haptoglobin-column reduces cytokine levels and protects against organ damage.

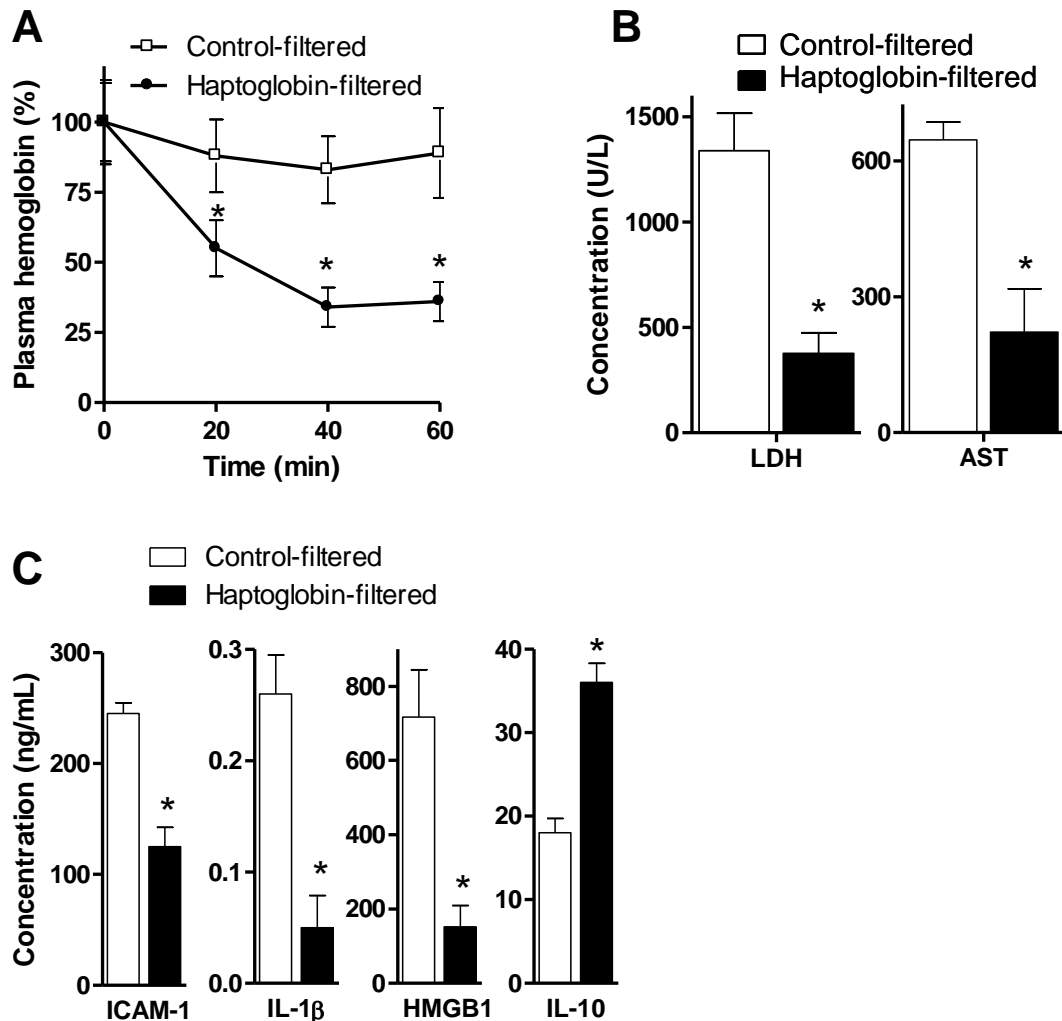


Figure 4-4. *In situ* filtration reduces (A) exogenous plasma hemoglobin and (B) markers of organ damage, and (C) dampens the inflammatory response after cecal ligation and puncture in rats. Sprague Dawley rats were connected to an extracorporeal circuit with a control- or haptoglobin column. Hemoglobin (27.5 mg/kg; saline) was injected into the circuit downstream of the column. Blood was then filtered *in situ* for 60 minutes at 1 mL/min, sampled pre-column at 5, 20, 40, and 60 minutes, and plasma hemoglobin measured spectrophotometrically. (A) Plasma hemoglobin as a function of time. Initial plasma hemoglobin concentration was 62 ± 6 mg/dL. *, $P < 0.05$ versus control; $n = 7-8$. Data presented as mean \pm SD. Cecal ligation and puncture was performed on control- and haptoglobin-filtered rats. Plasma was sampled at 24 hours and (B) ICAM-1, IL-1 β , IL-10, HMGB1, and (C) LDH, and AST were measured. *, $P < 0.05$ versus control-filtered; $n = 3$. Data presented as mean \pm SEM.

DISCUSSION

Conditions associated with increased plasma hemoglobin (cardiopulmonary bypass, burn trauma, stored red cell transfusion) are often complicated by the development of systemic inflammation [10]. Plasma hemoglobin increases the lethality of gram-negative bacteremia [21] and has been implicated in the development of other coagulatory and inflammatory disorders [22]. Removal of plasma hemoglobin may decrease inflammation and morbidity in these conditions.

Therapeutic removal of plasma hemoglobin by a sorption column requires efficient hemoglobin adsorption in the time it takes for blood to pass through the column. The haptoglobin-beads in a batch reactor (tube) exhibit logarithmic plasma hemoglobin removal, with a fitted removal rate ($\mu\text{g}/\text{sec}$) of $44\ln t$, where t is time in seconds ($R^2=0.94$) between 0 and 60 seconds. The rate at which the hemoglobin binds to the beads within the first 60 seconds is over three times greater than the rate post 60 seconds ($14\ln t$, $R^2=0.90$). The rate decrease in hemoglobin binding to the beads is due to the more readily available ligand (decorating the surface of the beads) becoming saturated, relegating further binding to the less accessible, intraporous ligand. In columns packed with these beads, the residence time of unbound plasma hemoglobin at a flow of 1 mL/min is approximately 60 seconds, indicating that the column packed with these beads can bind hemoglobin from plasma at expected flow. This is further indicated by the demonstration of efficient and predictable hemoglobin removal by the column over a range of flow conditions and hemoglobin concentrations (Fig. 4-1C-E).

While it is known that elevated plasma hemoglobin increases lethality during endotoxemia [11, 12], it was previously unknown whether elevated hemoglobin levels would increase lethality in a standardized model of polymicrobial sepsis induced by CLP. Here, we observe that hemoglobin dose-dependently increases lethality (Fig. 4-2B), and when hemoglobin is removed from solution by the haptoglobin column prior to administration into CLP challenged mice, sepsis-induced organ damage and mortality are reduced. It is of note that the CLP procedure performed was sublethal in the absence of exogenous hemoglobin and that the introduction of exogenous hemoglobin in the sham CLP group was asymptomatic. These data together indicate that extracellular hemoglobin, although well tolerated by healthy mice, significantly increase lethality in a polymicrobial sepsis model and that removal of hemoglobin with a haptoglobin column significantly reduces organ damage and death.

In situ blood filtration in a rat model of hemoglobinemia significantly reduces circulating plasma hemoglobin. The immunologic benefits of reducing plasma hemoglobin from septic rats with extracorporeal filtration include reducing subsequent circulating proinflammatory cytokines levels and increasing levels of the anti-inflammatory cytokine, IL-10 (Fig. 4-4C), which may act to suppress damaging proinflammatory cascades [23]. Organ protective effects of blood filtration with a haptoglobin column are evidenced by a reduction in plasma levels of enzymes (LDH and AST) typically elevated during tissue damage (Fig. 4-4B). The results suggest that it may be possible to develop a targeted approach to specifically and effectively remove hemoglobin through extracorporeal filtration.

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Chapter 5 Supplementary data

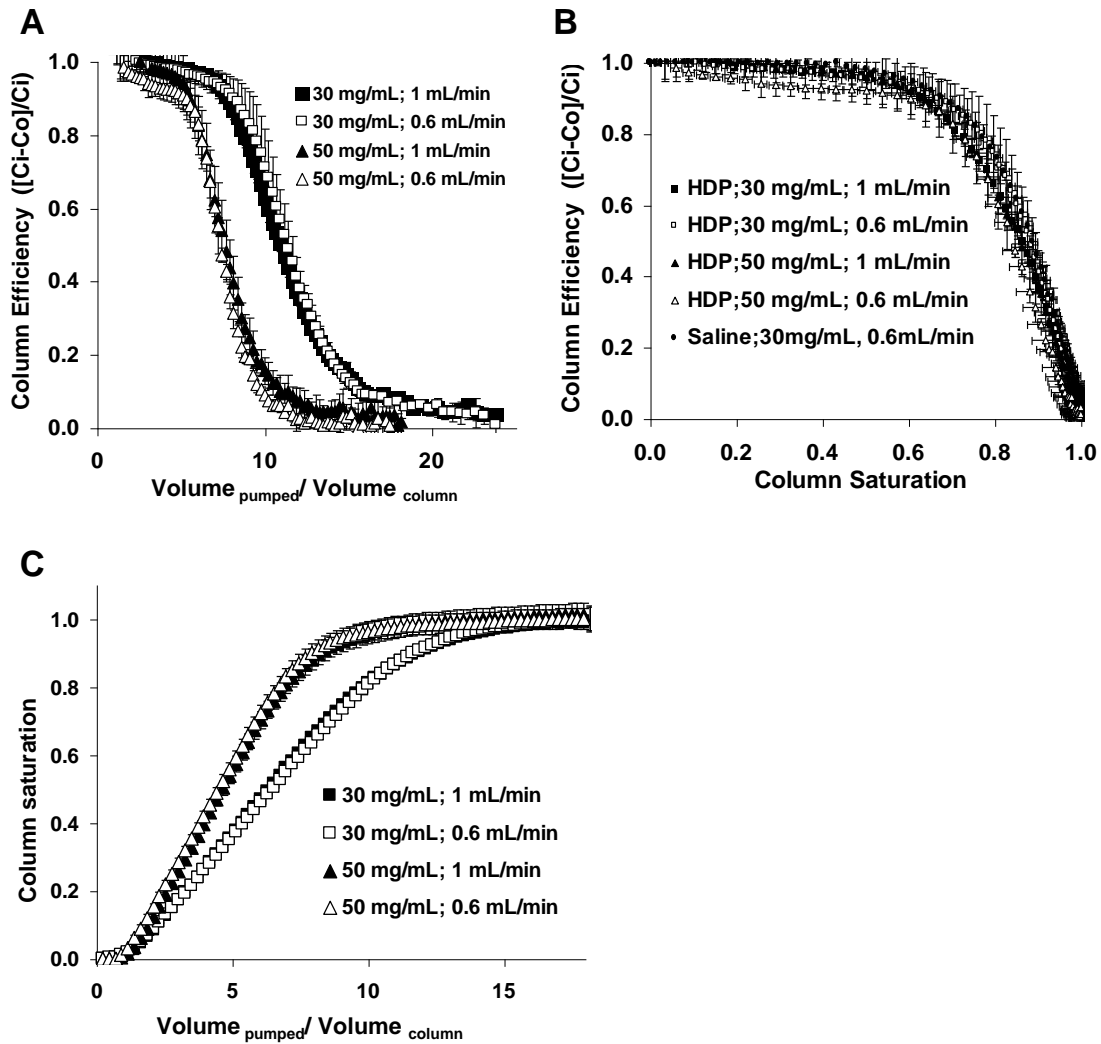


Figure 4-S1. Characterization of hemoglobin uptake by haptoglobin beads in flow-through conditions; Column efficiency vs. (A) volume pumped/volume column or (B) column saturation. (C) Column saturation vs. volume pumped/volume column. (A-C) Hemoglobin solutions of 50 and 30 mg/dL in (haptoglobin-depleted plasma and saline were pumped at 1 and 0.6 mL/min through the column (1.5 mL of haptoglobin-bead or control-beads) and collected in 0.35 mL fractions. The hemoglobin concentration in the effluent fractions was determined. The quantity of bound hemoglobin was calculated from the concentration difference at the inlet and outlet (effluent fractions) of the haptoglobin-column and corrected by the concentration difference at the inlet and outlet of the control-column. Column efficiency $([inlet\ concentration - outlet\ concentration] / inlet\ concentration; (C_o - C_i)/C_i)$ is demonstrated as a function of (A,C) volume pumped divided by volume of the column and (B) column saturation. Data presented as mean \pm SD. n=4

Development of animal model/surgical techniques

Direct hemoperfusion studies have previously been performed using rat models of sepsis [24]. All following animal manipulations have been approved by the North Shore-LIJ Health System IACUC (Protocol #2005-028).

Introduction

A rat cecal ligation and puncture (CLP) model was used to induce sepsis. In this model of fecal peritonitis, the cecum was devascularized, ligated such that intestinal contiguity remains intact, and perforated twice with a 22 gauge needle. Stool was then extruded into the peritoneum. CLP induces a polymicrobial peritonitis associated with the presence of devitalized tissue, as with human peritonitis, and as such this model is more clinically relevant than an endotoxemia model (CLP is currently the “gold standard” model of experimental sepsis[25]). The live pathogens also give a more relevant determinant for therapy than LPS alone [26]. To achieve a suitable survival (30-60%) of the control rats under these conditions, a bolus injection of antibiotic was provided at the time of CLP. This modification had been used successfully by the Tracey lab in the modulation of lethality in CLP mouse models and is more clinically relevant, as antibiotic use is a primary standard of care [27-29].

4.1 – Cecal ligation and puncture

Objective To develop a cecal ligation and puncture model for rats that produces a lethal dose (LD) of 30-60%.

Methods

Rats (250 to 300 g) were anesthetized with isoflurane inhalation (2-3%), and placed in the supine position. A 2 cm abdominal midline incision was performed and the cecum identified and externalized. A 4-0 silk suture was placed 1 cm proximal to the cecal tip, and tied down to occlude the cecal lumen. Using a needle, two separate enterotomies were made at the cecal tip. Next, using fine forceps, a piece of stool was extruded from a single cecal enterotomy into the peritoneum. The cecum was then returned to the abdominal cavity, and the abdominal wall closed primarily in two layers using running 4-0 Prolene sutures. Normal saline was administered subcutaneously immediately after the procedure to provide fluid resuscitation. Animals received subcutaneous Primaxin (antibiotic) and recovered on a warming blanket. Variables that were adjusted included the size of needle, the quantity of extruded stool, the volume of resuscitation fluid, and the quantity of antibiotics.

Results

During the early course of the design of the model, the lethality alternated between 0% and 100%. When a promising 50% LD was achieved through variable manipulation, the procedure was repeated 2 additional times. With 32 rats total, a CLP model was developed with a lethality of about 50%, see Fig. 4-S2.

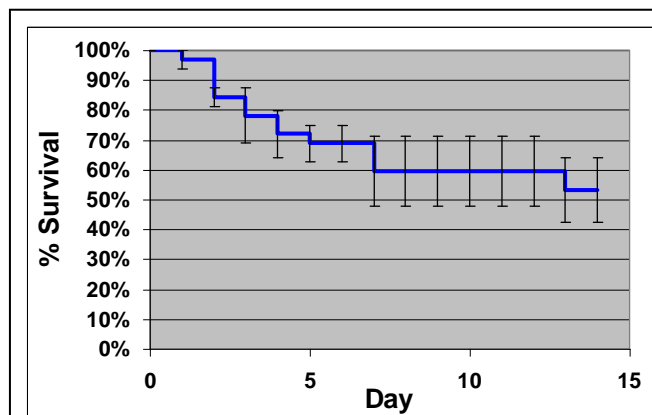


Fig. 4-S2. Rat survival after CLP. Cecal ligation and puncture was performed on 32 rats and survival was tracked. A lethal dose of approximately 50% was achieved (standard error displayed). n=32

Discussion

Numerous attempts were made to develop this model to the appropriate lethal dose. The finalized variables were the use of a 22-gauge needle, the extrusion of a 5 mm piece of stool, 3 mL/100 g body weight saline resuscitation, and 3 mg/kg antibiotics. This model produced a lethality of about 50%, without further surgical intervention.

4.2- plasma hemoglobin in untreated septic rats

Introduction

This thesis posits that septic rats have elevated plasma hemoglobin concentration, but little was known regarding the extent or time course of this elevation. These are important variables to understand before commencing hemoperfusion so that conditions such as hemoglobin-binding capacity of the filter and the timing of hemoperfusion can be estimated.

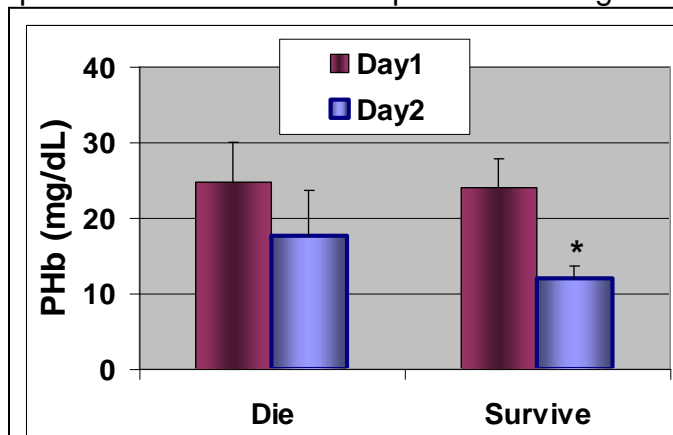


Fig. 4-S3. Surviving rats had lower plasma hemoglobin on Day 2 than on Day 1. Rats were subjected to CLP-induced sepsis and PHb measured over time. Rats were monitored for survival over two weeks. Values are presented as mean \pm SEM.

Objective To explore plasma hemoglobin concentration in septic rats over time.

Methods

Forty five male Sprague Dawley rats (275-325g, Taconic) were acclimated for at least 7 days prior to experimentation and anesthetized (isoflurane; 2.5%) prior to all manipulations. Sepsis was induced by cecal ligation and puncture (two punctures; 22 G needle) with a 5mm stool extrusion immediately followed by

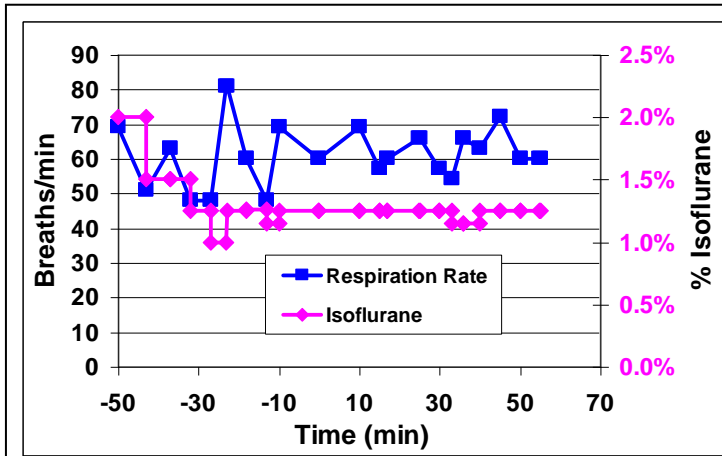


Fig. 4-S4 Monitoring respiration during hemoperfusion. The respiration rate was monitored during the hemoperfusion procedure in healthy rats and isoflurane anesthesia adjusted to maintain that rate between 45 and 100 Hz. Representative data from 1 rat displayed.

subcutaneous administration of antibiotic (Primaxin; 3 mg/kg) and saline for resuscitation (8 mL). Plasma hemoglobin was determined from blood sampled via retroorbital sinus. A maximum on two samples were taken per rat. Control samples (Day 0) were obtained from 8 rats prior to CLP. On successive days post-CLP, blood was sampled via retroorbital sinus, once per sinus, into heparinized tubes (n=43 on Day 1; n=20 on Day 2; n=6 on Day 3). Plasma hemoglobin concentration was determined spectrophotometrically as above. Animals were monitored for survival for two weeks. Two-tailed t-Test statistical analyses were carried out by comparing differences in plasma hemoglobin.

Results

Plasma hemoglobin concentration on days 0-3 are given in Figure 4-2a. Rats where plasma hemoglobin was measured for both day 1 and 2 post-CLP

were compared. These rats either survived for 14 days (n=12) or eventually succumbed to sepsis (“Die” group; n=6). The plasma hemoglobin for the “Survive” group decreased significantly between Day 1 and Day 2 (24 ± 4 vs. 12 ± 2 ; $p < 0.01$). The plasma hemoglobin for the “Die” group did not significantly decrease between Day 1 and Day 2 (25 ± 5 vs. 18 ± 6 ; $p > 0.35$). Values are presented as mean plus standard error (Fig. 4-S3).

Discussion

The plasma hemoglobin concentrations were significantly decreased between Day 1 and Day 2 in those rats that survived for 14 days post-CLP but not in those that succumbed to sepsis (Fig. 4-S3).

4.3 – Assess the effects of the surgical and perfusion procedures on healthy rats.

Introduction

During the development of the circuit for the hemoperfusion of septic rats, the circuit was tested on healthy rats to ensure that it was physically sound and biologically tolerated.

Objective To test the circuit in vivo to ensure that pressure limits are not exceeded, air did not become excessively entrapped, blood sampling was accomplished, hemolysis did not take place within the circuit, and that healthy animals survived the procedure.

Methods

Ten healthy rats were anesthetized with isoflurane, both femoral veins were cannulated using a 24-gauge angiocatheter and heparinized (1u/10gr). The filter of Chapter 2 Fig.2-2 was filled with 0.7 mL of haptoglobin-free beads and inserted in a circuit similar to that shown in Chapter 2 Fig.2-4. The sterilized circuit was primed with heparinized saline (10 units/mL) and connected to the venous cannulae. Pressure transducers where

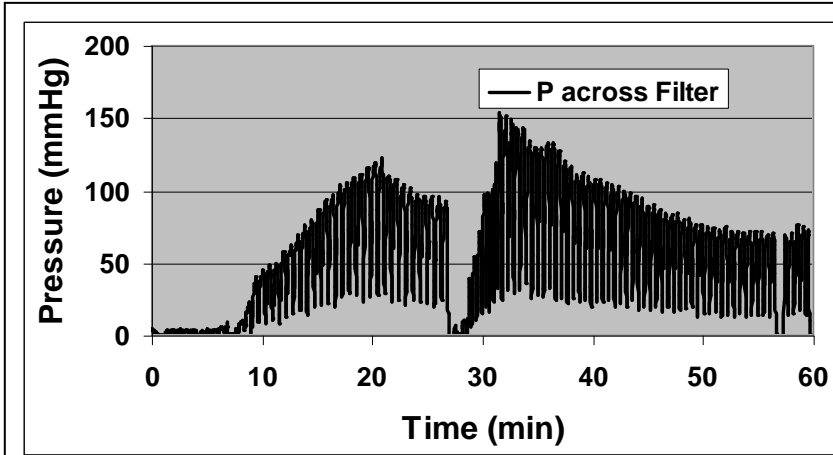
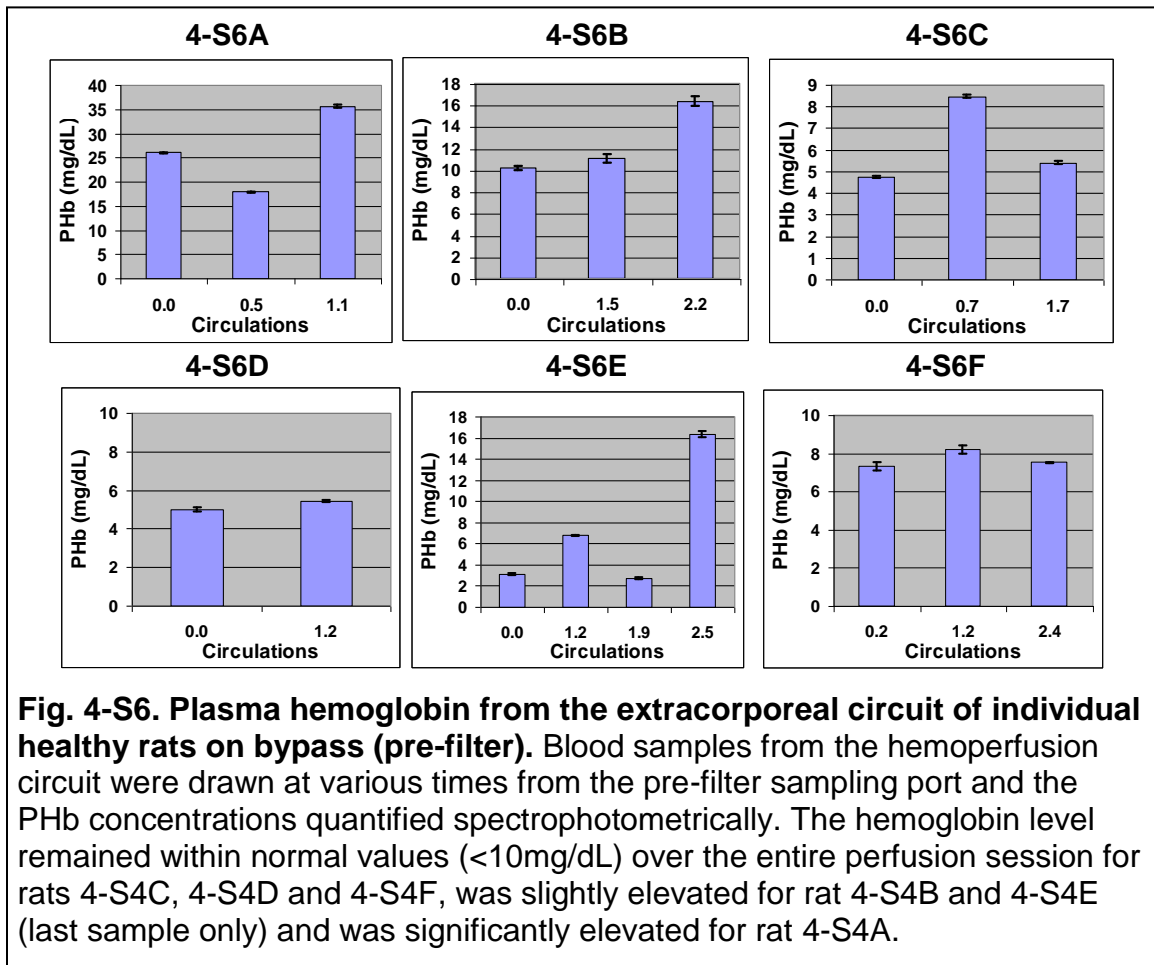


Fig. 4-S5. Pressure across filter during hemoperfusion. Pressures pre- and post-filter were monitored via computer-interfaced sensors to ensure that the trans-filter pressure did not exceed 350 mmHg. Displayed is a representative pressure tracing during a hemoperfusion session without major air accumulation in the filter. Note the drop in pressure at 28 and 58 minutes are artifacts due to blood sampling.

integrated into the circuit via pressure ports and read into a computer via I/O



card. Pump flow was maintained at 0.75 mL/min for 60 min. Blood was collected pre- and post-filter via sampling ports, and the plasma isolated and assayed for plasma hemoglobin as described above. The sampling ports were rinsed and dried after each blood sample was taken. Respiration rate was monitored manually and anesthesia adjusted to maintain a rate between 45 and 100 Hz. At the end of the hour, the blood in the circuit was returned to the rat by flushing it with saline, the cannulae removed, the veins ligated, the wound sutured, and the rats recovered on a heating pad. No resuscitation fluid or antibiotics were administered.

Results

All ten rats quickly awakened from anesthesia. Though some exhibited piloerection and some were slightly sluggish for a couple of hours, by 24 hours all were indistinguishable from naïve rats (aside from sutures). All survived to three weeks. The respiration rate was maintained between 45 and 100 Hz (mostly between 50 and 70 Hz; Fig 4-S4) through careful monitoring and adjusting the concentration of anesthetic inhalant.

Figure 4-S5 displays a standard pressure profile over the course of the hemoperfusion procedure. The pressure across the filter represents the difference between the pre- and post- filter pressures. Here it is maintained below 200 mmHg, and mostly below 150 mmHg, which corresponds to a shear rate of approximately 3000 sec^{-1} which should result in minimal hemolysis [30] (for derivation, see section 1.3 and Appendix B). At 200 mmHg, the shear stress is half of that seen physiologically within blood vessels [31]. The chart displayed in Figure 4-S6 shows the plasma hemoglobin concentration, taken from the circuit of six healthy rats while on bypass. The data show that the plasma hemoglobin of the circuit remain within normal values ($<10\text{mg/dL}$, see Chapter 5, Figure 2a, time “0”) over the one hour perfusion for rats 4-S6C, 4-S6D and 4-S6F. Plasma hemoglobin was slightly elevated for rat 4-S6B and 4-S6E (last sample only) and was significantly elevated for rat 4-S6A.

Discussion

The chart displayed in Figure 4-S5 shows that we can maintain the pressure within the circuit to within a reasonable range to protect the red blood

cells from shear-induced lysis. The fluctuations which occur at about 2 Hz represent the pulsatile flow produced by the pump as its rollers engage the tubing within the raceway. Note that these rats were perfused using a 3/16" ID filter housing. The design of the housing has since changed to a 1/4" ID filter housing which theoretically decreased the pressure drop by 68% (see Appendix B).

The need to carefully monitor respiration rate has been important. Over the course of the 1-2 hours that the rat is anesthetized (note x-axis in Fig. 4-S4 reads from -50 minutes before perfusion commencement) the anesthetic can build up in the body, causing a depression in the rat's respiration that can be fatal.

Figures 4-S6C, 4-S6D, and 4-S6F show no appreciable increase in plasma hemoglobin concentration over the course of the hour-long hemoperfusion session. The ability to achieve perfusion with negligible hemolysis was a major concern, as hemolysis within the pump head or the filter may reduce the efficiency of, or even completely saturate the filter. The proper cleaning of the sampling ports was found to be essential to accurate measurement of plasma hemoglobin. Hemolysis occurred when blood was allowed to pool and dry in the port, which resulted in artificially high plasma hemoglobin measurements (Fig 4-S6A). It is also possible that elevated pressures at the pump inlet may contribute to the elevated plasma hemoglobin in some of the rats as well. Lastly, we see from this data that healthy rats were perfused with a filter filled with haptoglobin-free beads without showing evidence

of toxicity due to the beads or ill effects due to the perfusion and surgical procedure.

4.4 Rebound of plasma hemoglobin

Objective To measure the plasma hemoglobin level in septic rats 24 hour post-filtration.

Method

Blood was sampled at 24 h post- filtration and CLP as previously described and plasma hemoglobin measured. Levels of plasma hemoglobin in rats filtered with haptoglobin- or control-filters were compared by t-test with a confidence of 95%.

Results

Plasma hemoglobin in rats filtered with haptoglobin-filters (14 ± 1.5 mg/dL) was not significantly lower than in rats filtered with control-filters (27 ± 11 mg/dL; $n=3$; $p=0.37$). Data presented at mean+SEM. (Fig. 4-S7)

Discussion

There was a significant decrease in exogenous plasma hemoglobin in rats filtered with haptoglobin-filters as compared to rats filtered with control-filters over the course of the filtration procedure (Chapter 4, Figure 4-4a), but there was no significant difference in plasma hemoglobin levels by 24 hours post- filtration and CLP. However, there was a trend toward reduced plasma hemoglobin at 24 hours post- haptoglobin-filtration as compared to control-filtration. This reduction may be significant in a larger sample.

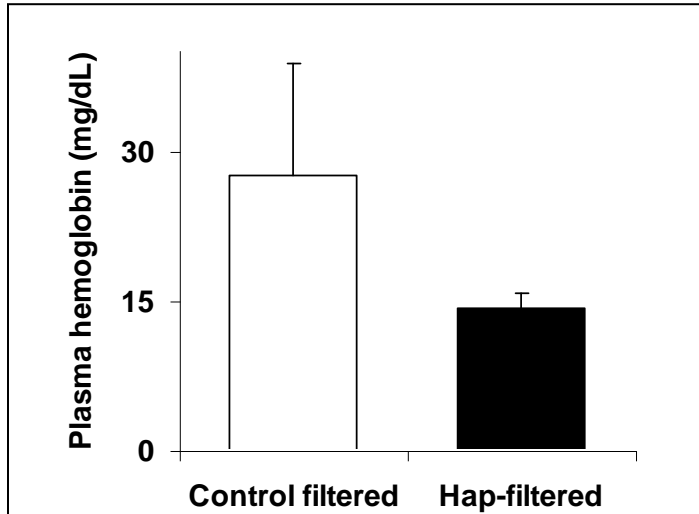


Fig. 4-S7. Plasma hemoglobin concentrations at 24 h post-filtration.

Plasma hemoglobin concentrations at 24 h post filtration in rats filtered with haptoglobin- or control-filters are not significantly different at 24 h post-filtration. n=3.

Chapter 5

Conclusion

A platform was developed for the specific removal of toxin through an extracorporeal blood-contacting device. The tailoring of the column-based platform to remove hemoglobin from plasma using haptoglobin-bound beads was described. The removal of hemoglobin in the context of sepsis was explored, as recent evidence suggested that plasma hemoglobin mediates lethality during sepsis.

The kinetics of hemoglobin removal for this device were established. The use of breakthrough analysis to measure the hemoglobin-binding characteristics of the column led to accurate modeling of hemoglobin removal. It was suggested that this technology can be applied to other toxins, and that any new configuration be assessed by breakthrough curve analysis to predict operational parameters.

Filtration of hemoglobin *ex vivo* led to decreased markers of organ damage and lethality in a model of mouse sepsis with hemoglobinemia as well as reduced proinflammatory responses in macrophage cultures. Filtration of hemoglobin *in situ* reduced extracellular hemoglobin in rats and led to reduced organ damage markers and proinflammatory cytokines in the plasma of septic rats. These together indicated that the removal of plasma hemoglobin during sepsis was therapeutic.

Future directions

Filtration of plasma hemoglobin from rats *in situ* reduced cytokines and protected organs, but increased survival was not demonstrated due to surgical difficulties and lack of statistical power. Additional animals can be filtered as in

Chapter 4 to demonstrate significance in survival between rats filtered with haptoglobin-columns and control-columns.

The efficacy of hemoglobin removal through *in situ* filtration of animals already septic has not yet been demonstrated. This can be accomplished by filtering the blood of septic rats 24 hours post-CLP with the haptoglobin beads. For that study to succeed, a couple of technical hurdles need to be overcome. At 24 hours post-CLP, the plasma volume of septic rats is decreased and the hematocrit increased roughly 25% (unpublished data), making hemoperfusion more difficult and dangerous to the rats. Intravenous injection (0.5 mL/min) of about 5 mL saline/rat before blood is introduced to the circuit should make the procedure more manageable. A second issue to be addressed is that the variability in initial plasma hemoglobin levels between septic rats may preclude statistically meaningful results. This may be managed by measuring the initial plasma hemoglobin level of the rat within the first few minutes of the procedure and adding exogenous hemoglobin to the circuit post-filter to a certain final concentration in the plasma.

Although there is now recognition of the danger of plasma hemoglobin in sepsis, there has not been a prospective clinical study to measure plasma hemoglobin and correlate it to sepsis severity. A next step for this body of work is to perform a clinical study to measure plasma hemoglobin in septic patients. This study would draw blood from a central line to avoid hemolysis, and correlate levels of plasma hemoglobin with cytokines, markers of organ damage, and clinical outcome.

To improve the scalability of the system, haptoglobin analogues with similar affinity to hemoglobin can be developed to simplify manufacture of columns. Additionally, the attachment of haptoglobin or its analogues to dialysis fibers as an alternative to column-based hemoperfusion can be explored.

Back to the clinic

If removal of hemoglobin from plasma with the extracorporeal device continues to prove therapeutic, it would be a simple matter to employ it clinically. The device can be implemented in a manner similar to dialysis, but in a simpler format; it would only require a blood-contacting surface and not a dialysate surface as well. Designs that bind the haptoglobin or its analogue to current clinically used and very inexpensive dialyzers can be explored. Should the developed device prove successful, then its use can be easily adaptable to current dialysis systems and their disposables. Only the dialyzer unit itself would have to be replaced and perhaps flow conditions adjusted. The presence of such a large base of dialysis systems in the health centers, systems that health providers are comfortable using, can allow a proven filter to be readily accepted into clinical use without the need for capital expenditure or much training.

Additionally, the device may be used as a post-fit module for existing extracorporeal units, both in sepsis, where dialysis is often necessary due to acute renal failure (23%-51% of cases) [1] and in non-sepsis related procedures where incidental plasma hemoglobin may contribute to systemic inflammation (Chapter 1).

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Appendix A- Derivation of plasma hemoglobin removal model

[Rate of Change] = [Rate of Change in] – [Rate of Change out]

$$\frac{\partial C(t)}{\partial t} = C(t) * \left(\frac{Q}{V}\right) * (1-E) + \frac{G}{V} + \frac{H(C)}{V} - \frac{R(C)}{V} - C(t) * \left(\frac{Q}{V}\right) = C(t) * \left(\frac{Q}{V}\right) * [(1-E)-1] + \frac{G}{V} + \frac{(H-R)(C)}{V} \Rightarrow$$

$$\partial C(t) = \left[-C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] + \frac{G}{V} \right] \partial t; \frac{H-R}{V} = 0$$

$$\frac{\partial C(t)}{\left[C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} \right]} = -\alpha \Rightarrow$$

$$\int \frac{\partial C(t)}{\left[C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} \right]} = -\int \partial t \Rightarrow$$

$$\frac{1}{\left(\frac{Q}{V}\right) * [1-(1-E)]} * \ln \left[C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} \right] = -t + b \Rightarrow$$

$$\ln \left[C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} \right] = -\left(\frac{Q}{V}\right) * [1-(1-E)] * t + b \Rightarrow$$

$$\exp \left\{ \ln \left[C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} \right] \right\} = \exp \left\{ -\left(\frac{Q}{V}\right) * [1-(1-E)] * t + b \right\} \Rightarrow$$

$$C(t) * \left(\frac{Q}{V}\right) * [1-(1-E)] - \frac{G}{V} = b * \exp \left\{ -\left(\frac{Q}{V}\right) * [1-(1-E)] * t \right\}$$

$$C(t) = \frac{1}{\left(\frac{Q}{V}\right) * [1-(1-E)]} * \left[\frac{G}{V} + b * \exp \left\{ -\left(\frac{Q}{V}\right) * [1-(1-E)] * t \right\} \right]$$

At initial condition $t=0$, $C(0)=C_0$

$$C(0) = C_0 = \frac{1}{\left(\frac{Q}{V}\right) \times [1 - (1 - E)]} * \left[\frac{G}{V} + b \right] \Rightarrow$$

$$b = C_0 \left(\frac{Q}{V} \right) \times [1 - (1 - E)] - \left[\frac{G}{V} \right]$$

$$C(t) = \frac{1}{\left(\frac{Q}{V}\right) * [1 - (1 - E)]} * \left[\frac{G}{V} + \left[C_0 \left(\frac{Q}{V} \right) \times [1 - (1 - E)] - \left[\frac{G}{V} \right] \right] * \exp \left\{ - \left(\frac{Q}{V} \right) * [1 - (1 - E)] * t \right\} \right]$$

or

$$C(t) = C_0 * e^{\left\{ - \left(\frac{Q}{V} \right) * E * t \right\}} + G * \frac{1}{Q * E} * \left[1 - e^{\left\{ - \left(\frac{Q}{V} \right) * E * t \right\}} \right]$$

Appendix B- Pressure Drop and Shear Predictions- L/D of 12, 5, and 3

| | | | | |
|-------------------------------|--|----------------|----------------|-------------------------|
| Column Diameter | 0.1875 | 0.25 | 0.3125 | inch |
| Volume of drained beads | 1 | 1 | 1 | mL |
| Column Height (L) | 2.21 | 1.24 | 0.80 | inch |
| Flow (Q) | 1 | 1 | 1 | mL/min |
| Sphere (Bead) Diameter (Ds) | 90 | 90 | 90 | µm |
| Column Diameter | 4.8E-03 | 6.4E-03 | 7.9E-03 | m |
| Column Crossectional Area | 1.8E-05 | 3.2E-05 | 4.9E-05 | m ² |
| Column Height (L) | 5.6E-02 | 3.2E-02 | 2.0E-02 | m |
| Flow (Q) | 1.7E-08 | 1.7E-08 | 1.7E-08 | m ³ /sec |
| Sphere Diameter (Ds) | 9.0E-05 | 9.0E-05 | 9.0E-05 | m |
| Total Volume of column | 1.0E+00 | 1.0E+00 | 1.0E+00 | cm ³ |
| Total Volume of column | 1.0E-06 | 1.0E-06 | 1.0E-06 | m ³ |
| Void Fraction (ε) | 0.36 | 0.36 | 0.36 | ---- |
| Void Volume of column | 3.6E-07 | 3.6E-07 | 3.6E-07 | m ³ |
| Superficial Velocity (v') | 9.4E-04 | 5.3E-04 | 3.4E-04 | m/s |
| Volume of Sphere | 3.8E-13 | 3.8E-13 | 3.8E-13 | m ³ |
| Volume of beads in column | 6.4E-07 | 6.4E-07 | 6.4E-07 | m ³ |
| Volume of beads in column | 6.4E-01 | 6.4E-01 | 6.4E-01 | cm ³ |
| Number of beads in column | 1.7E+06 | 1.7E+06 | 1.7E+06 | beads |
| Surface area of bead | 2.5E-08 | 2.5E-08 | 2.5E-08 | m ² |
| Surface area of all beads | 4.3E-02 | 4.3E-02 | 4.3E-02 | m ² |
| Surface area of column | 8.4E-04 | 6.3E-04 | 5.0E-04 | m ² |
| Dynamic Viscosity (μ): Plasma | 1.6E-03 | 1.6E-03 | 1.6E-03 | Kg/m*s |
| Dynamic Viscosity (μ): Blood | 4.0E-03 | 4.0E-03 | 4.0E-03 | Kg/m*s |
| Density of liquid (ρ): Plasma | 1025 | 1025 | 1025 | kg/m ³ |
| Density of liquid (ρ): Blood | 1060 | 1060 | 1060 | kg/m ³ |
| Pressure Drop (ΔP) | $\Delta P = \frac{150 \mu v' L}{D_S^2} \frac{(1 - \epsilon)^2}{\epsilon^3} + \frac{1.75 \rho v'^2 L}{D_S} \frac{(1 - \epsilon)}{\epsilon^3}$ | | | |
| Pressure Drop: Plasma | 1.4E+04 | 4.3E+03 | 1.8E+03 | Kg*m/s ² |
| Pressure Drop: Plasma | 103 | 32 | 13 | mmHg |
| Pressure Drop: Blood | 3.4E+04 | 1.1E+04 | 4.4E+03 | Kg*m/s ³ |
| Pressure Drop: Blood | 256 | 81 | 33 | mmHg |
| Pressure Drop: Blood | 256 | 81 | 13 | mmHg |
| Pressure Drop: Blood | 3.4E+04 | 1.1E+04 | 1.8E+03 | N/m ² |
| Force from Pressure | 6.1E-01 | 3.4E-01 | 8.8E-02 | N |
| Shear stress (τ) | 1.4E+01 | 7.9E+00 | 2.0E+00 | N/m ² |
| Shear rate (γ) | 3.5E+03 | 2.0E+03 | 5.1E+02 | sec⁻¹ |
| shear rate squared | 1.2E+07 | 3.9E+06 | 2.6E+05 | sec ⁻² |