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Development of a translational animal model of sepsis

Nathaniel E. Hayward
The University of Western Ontario

Supervisor
Dr. Christopher G Ellis
The University of Western Ontario

Joint Supervisor
Dr. Dwayne N Jackson
The University of Western Ontario

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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DEVELOPMENT OF A TRANSLATIONAL ANIMAL MODEL OF SEPSIS

(Thesis format: Integrated-Article)

by

Nathaniel E. Hayward

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Sepsis is an excessive inflammatory response to infection that leads to multiple organ failure. The high mortality rates in the intensive care unit have remained stagnant, which can be attributed to the disconnect between the bench and the bedside. There is a global need for an animal model of sepsis that is more relevant to the clinical scenario. We developed an in vivo rat model of sepsis, with a high level of instrumentation, to monitor macrovascular and microvascular changes over the course of a feces-induced peritonitis (FIP).

In addition, early fluid resuscitation of septic patients is associated with better clinical outcomes; however, the type of fluid used for resuscitation is highly disputed. The first application of our in vivo model was to use clinical resuscitation targets to test the effect of fluid type (crystalloid or colloid) on both the microvasculature and the systemic circulation.

Keywords: sepsis, feces-induced peritonitis, colloid, crystalloid, microcirculation
Co-Authorship Statement

A version of Chapter 2 is in preparation for submission entitled “Development of a translational animal model of sepsis” by Nathaniel E Hayward, Dr. Michael D Sharpe, Dr. Dwayne N Jackson, and Dr. Christopher G Ellis to BioMed Research International. Experiments, data collection, validation, and analysis were conducted by myself. The experimental protocol was based upon input from Dr. Sharpe, while Dr. Jackson, and Dr. Ellis provided experimental guidance.

Chapter 3 is entitled “Assessing fluid resuscitation efficacy in a translational animal model of sepsis” by Nathaniel E Hayward, Dr. Michael D Sharpe, Dr. Alison Fox-Robichaud, Dr. Dwayne N Jackson, and Dr. Christopher G Ellis. Experiments, data collection, validation and analysis were conducted by myself. The resuscitation protocol was based on input from Dr. Sharpe, and Dr. Fox-Robichaud, while Dr. Jackson, and Dr. Ellis provided experimental guidance.
“Tiger got to hunt, bird got to fly; Man got to sit and wonder ‘why, why, why?’

Tiger got to sleep, bird got to land; Man got to tell himself he understand."

- Kurt Vonnegut, Cat’s Cradle, 1963
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<td>Cecal Ligation and Perforation</td>
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<td>CO</td>
<td>Cardiac Output</td>
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<tr>
<td>COP</td>
<td>Colloid Osmotic Pressure</td>
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<tr>
<td>CVP</td>
<td>Central Venous Pressure</td>
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<td>D\l\IVVM</td>
<td>Dual-wavelength intra-vital video microscopy</td>
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<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
</tr>
<tr>
<td>FCD</td>
<td>Functional Capillary Density</td>
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<tr>
<td>FIP</td>
<td>Feces-Induced Peritonitis</td>
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<tr>
<td>FOV</td>
<td>Field of View</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
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<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
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<td>MAP</td>
<td>Mean Arterial Pressure</td>
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<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
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<tr>
<td>SDF</td>
<td>Side-stream dark field</td>
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<tr>
<td>SO₂</td>
<td>Oxygen Saturation of Red Blood Cell</td>
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<tr>
<td>SV</td>
<td>Stroke Volume</td>
</tr>
<tr>
<td>TPR</td>
<td>Total Peripheral Resistance</td>
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<td>WBC</td>
<td>White Blood Cell</td>
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Chapter 1

Introduction and Background

1.1 Thesis Outline

This thesis was motivated by the needs of ICU physicians, and sepsis researchers alike, to provide a well-controlled animal model that is representative of human sepsis. The disparity between the bench and the bedside has been brought to light recently, due to the failure of several clinical trials aimed at improving the survivability of sepsis. The aim of this model was to provide a platform to test common ICU interventions, and attempt to explicate the impact of these interventions on the microvascular environment surrounding the vital organs. The difficulties in developing a working animal model of sepsis that has clinical applications furthered our understanding of the complexity of the sepsis state, and made clear the challenges of translational research. These difficulties are minor, however, when compared to the difficulty in understanding the human sepsis condition given the multitude of potentially confounding variables, routes of infection, and patient backgrounds.

This thesis is divided into four chapters. The first chapter provides an introduction to the current state of sepsis in the ICU, and the issues surrounding translating bench-side research to clinical practice. It also summarizes current therapies aimed at restoring macrovascular and microvascular circulation. This chapter further outlines the issues with current animal models of sepsis, and highlights some important aspects of an “ideal” translational model.

The second chapter outlines the development of an animal model of sepsis that is more clinically relevant. In collaboration with ICU physicians, we demonstrate our two-sided (macrovascular and microvascular) instrumentation techniques, which provide data similar to what would be available in the critical care environment. To illustrate the similarity in acquired measurements, this chapter also shows sample data collected from a septic animal.
The third chapter of this thesis demonstrates an application of our model addressing a common debate among clinicians as to which fluid should be used for resuscitation. This experiment was guided by clinical resuscitation targets, and aimed to elucidate the mechanistic effect of different fluids on microvascular restoration in sepsis. Two of the most common fluids used clinically, saline and albumin, were tested as the first application of the model. This chapter provides preliminary data for each fluid type used for resuscitation, and illustrates the associated microvascular effect of achieving hemodynamic optimization targets.

The final chapter provides a critical discussion of experimental findings, and explores potential additions to improve the fidelity of the model. Lastly, this chapter explores possible future applications of our model.

### 1.2 Sepsis

Sepsis is a systemic inflammatory response to infection that causes a wide range of clinical conditions in an unpredictable manner. The pathogenesis of sepsis can involve multiple organ systems, and is characterized by an initial inflammatory phase comprised of increased cardiac output, low systemic vascular resistance, and heterogeneity of microvascular blood flow, depleted blood volume due to increased vascular permeability, and decreased blood pressure\[1, 2\]. This is followed by an immunosuppression phase, which involves decreased cardiac output, low systemic vascular resistance, and respiratory distress in tissue cells, tissue edema, and indications of hypoxia within the vital organs. The progression of sepsis is rapid, the symptoms between patients are inconsistent, the site of infection is variable, and the timing of intervention is critical to survivability\[3-6\]. For this reason, despite major improvements in the quality of patient care, sepsis mortality rates have remained stagnant.

A landmark epidemiology study analyzing the healthcare burden of severe sepsis in the United States, demonstrated that the cost of sepsis to the healthcare system in 1995 was $16.7 billion annually, with over 800,000 diagnosed cases. \[5\]. With an associated mortality rate of 30%, sepsis was the leading cause of death in the intensive care unit. Despite an increased knowledge of the pathophysiology of sepsis, the mortality rate of
patients with severe sepsis remains stagnant. Over 70 multi-center clinical trials have been implemented, aimed at improving the survivability of sepsis, and most of them failed to show a positive survival benefit[7]. This can be attributed to the difficulty in defining sepsis, and the degree of disconnect between the bench and the bedside[7, 8]. It has also recently been recognized that consideration of the microcirculation in sepsis may lead to improved survivability[9].

### 1.3 The Microcirculation in Sepsis

The microcirculation (vessels <100µm in diameter) is responsible for transporting oxygen and nutrients to tissue cells and, if needed, delivers therapeutics. This network of arterioles, capillaries, and post-capillary venules form the microcirculatory unit that is severely affected in sepsis. Within this network, endothelial cells line the inside of the vessels, and smooth muscle cells regulate arteriolar tone, while red blood cells, leukocytes, platelets and plasma form the components of the blood[10]. The transport of oxygen occurs primarily at the capillary level, and involves passive diffusion of oxygen from the arterioles and the capillaries to the surrounding environment[11]. This system is highly regulated and, in healthy individuals, has the ability to distribute blood flow to meet the unique oxygen requirements of each tissue. In sepsis, the microvascular system is compromised, and oxygen transport to the vital organs is severely affected. The restoration of tissue perfusion is intimately linked to survival in sepsis[12].

The microcirculatory alterations common in sepsis include: an increase in the amount of stopped flow capillaries, maldistribution of oxygen delivery to tissue, increases in arteriolar diameter, and increased spatial distance between perfused capillaries for oxygen diffusion[1, 13, 14]. Within this network, endothelial cell swelling, leukocyte trafficking and stiffness, disruption of vascular tone regulation, stiff red blood cells, and fibrin/platelet clotting can lead to stopped flow in capillaries, and further compromised tissue function[15, 16].

The early septic response in humans is associated with a hypodynamic phase in which there is an imbalance between the oxygen demands of tissue, and the necessary supply to maintain proper organ function. This leads to global tissue hypoxia, and the recognition
of this phase incites early goal-directed therapy implementations aimed at optimizing systemic hemodynamic parameters in an attempt to restore tissue oxygenation[3]. Despite this optimization, microcirculatory perfusion can remain impaired [17, 18]. It follows that compromised microvasculature common to sepsis, and the associated hypoxic conditions in tissue, initiates a cascade of pathogenic mechanisms that could eventually result in organ failure[10]. The relationship between impaired oxygen extraction and mortality in sepsis has been known for decades; however, the emergence of bedside technology allowing for the observation of patient microcirculation has illustrated the importance of considering the microcirculatory environment given the pivotal role it plays in sepsis-induced organ dysfunction [10, 12, 15, 16, 19]. For this reason, goal-directed therapies aimed at re-establishing macrovascular homeostasis exclusively may be insufficient in restoring microcirculatory flow in the tissues of septic patients and thus do not prevent organ failure [20, 21].

It is well known that heterogeneous distribution of blood flow in sepsis causes some capillaries to be under perfused, while some capillaries remain normal or highly perfused[13]. This maldistribution of blood flow greatly increases the diffusion distance of oxygen in the capillary bed, which can quickly cause hypoxic conditions. The low levels of mixed-venous oxygen saturation, and high lactate levels at the onset of sepsis, are indicative of this response. The production of lactate is indicative of anaerobic metabolism, which is related to tissue hypoxia, due to decreased oxygen extraction[22]. Lactate could also increase due to mitochondrial failure, or due to abnormalities in pyruvate dehydrogenase activity[23, 24]. The decrease in oxygen extraction ratio despite increased cardiac output in the hyperdynamic phase of sepsis could be due to impaired mitochondrial respiration, reducing the utilization of available oxygen[25]. On the other hand, it could also be due to heterogeneity of flow caused by microvascular injury, which causes inadequate oxygen supply to some regions of tissue[1, 26].

Endothelial cells play an important role in sensing flow, and have the ability to transmit upstream information about the downstream microcirculatory conditions[27]. These cells also have regulatory properties that control microvascular tone, permeability and capillary recruitment, as well as controlling coagulation and immune function[28]. In
sepsis, the endothelial cells do not function properly, which causes loss of cell-to-cell communication and smooth muscle control. The dysfunction of endothelial cells in sepsis thus has a marked effect on microcirculatory perfusion. Endothelial-cell-mediated microvascular dysfunction causes arteriolar vasodilation, leukocyte adhesion, microvascular permeability, compromised cell-to-cell communication, altered functional capillary density, maldistribution of capillary blood flow, and abnormal oxygen transport and tissue oxygenation[1, 10, 20, 27]. Furthermore, erythrocytes are also affected in sepsis and become less deformable, which affects their ability to release nitric oxide in the presence of hypoxia to cause a vasodilatory response[29]. Taken together, the microvascular environment is dysfunctional, leading to a severe decrease in peripheral vascular resistance, tissue edema, myocardial and multiple organ microvascular dysfunctions.

It is not clear whether or not therapeutic interventions (e.g. fluid resuscitation, vasodilators, vasopressors) aimed at improving global oxygen delivery to tissue will be effective if there is not an increase in microvascular recruitment and flow. A major focus of this thesis was to utilize imaging techniques to quantify the degree of microvascular failure in the capillary bed of an animal throughout the onset of an applied septic insult. Dual-wavelength intra-vital video microscopy provided the unique ability to measure velocity, supply rate, hematocrit, and oxygen saturation for single capillaries, which illustrates the mechanistic effect of microvascular dysfunction in sepsis.

1.3.1 Microcirculatory Monitoring in the ICU

It is well established that a poorly functioning microvascular system leads to multiple organ failure and poor prognosis in sepsis[30]. Despite therapies aimed at re-establishing macrovascular homeostasis and improving global oxygen delivery (fluid resuscitation, vasopressors, inotropic agents, vasodilators, blood transfusions), the inability to re-perfuse hypoxic capillary beds renders these interventional techniques ineffective[10, 31]. The ability to assess the degree of microvascular dysfunction in the ICU is thus a valuable tool to provide insight into the severity of sepsis, and the environment surrounding the vital organs[32].
Laser-Doppler, and forearm ischemia reperfusion techniques can measure regional microvascular alterations; however, these methods do not take heterogeneity of flow into account, which leads to underestimation of the severity of microvascular dysfunction in sepsis[33]. In order to address the need for observation of the microvasculature at the bedside, side-stream dark field (SDF) imaging was developed and can be implemented in critically ill patients[10]. This technique consists of a 530-nm light source, a wavelength that is absorbed by hemoglobin within erythrocytes, allowing for the viewing of flowing cells in the sublingual vascular bed. The images obtained using SDF imaging can be analyzed post capture to determine the severity of microvascular dysfunction. Briefly, the images are separated into quadrants and capillaries are categorized based on RBC transit to obtain a measure of perfusion quality. In addition, dividing the image by 3 vertical and 3 horizontal lines, and determining the number of vessels that cross these lines, is a determinant of vessel density[34].

While SDF imaging has demonstrated correlations with severity in sepsis, it is not without limitations. The measurements made using this technique illustrate the degree of perfusion within the observed network; however, there has been no demonstrated ability to extract oxygen saturation data within the perfused capillaries. Furthermore, the technique is very sensitive to motion and pressure artifacts, and it is thus difficult to observe the exact same network over a period of time[34]. Also, the wavelength of light used in this technique does not allow for good contrast between the red blood cells and the capillary lumen[11], which is subject to inter-observer variability due to qualitative assessment of perfusion. The relevance of alterations in sublingual perfusion in a heterogeneous disease state such as sepsis have not been shown to be a definitive indicator of septic onset, and until more objective measurement tools are available, should only act as a secondary marker of the progression of microvascular failure in sepsis.
1.4 Overview of Clinical Sepsis Diagnostic Criteria

One of the major challenges in diagnosing sepsis in global ICUs has been due to lack of a precise definition of sepsis[6]. In addition, patients in the ICU often suffer from comorbidities, which cause a largely heterogeneous array of symptoms.

Table 1-1 provides the criteria most commonly used to diagnose systemic inflammatory response, and illustrates the flow of criteria used to identify sepsis.
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<td>Core temperature ≥38°C or ≤36°C</td>
<td></td>
</tr>
<tr>
<td>Heart rate ≥ 90 beats/min</td>
<td></td>
</tr>
<tr>
<td>Respiratory rate &gt;20 breaths/min or PaCO$_2$ &lt;32 mmHg</td>
<td></td>
</tr>
<tr>
<td>White blood cell count ≥ 12,000 cells/mm$^3$ or ≤ 4,000 cells/mm$^3$ or differential showing 10% immature (band) neutrophils</td>
<td></td>
</tr>
<tr>
<td>Sepsis: 2 or more of SIRS + proven/suspected infection</td>
<td></td>
</tr>
<tr>
<td>Documented infection</td>
<td></td>
</tr>
<tr>
<td>Perforated viscous</td>
<td></td>
</tr>
<tr>
<td>Syndrome associated with a high likelihood of infection</td>
<td></td>
</tr>
<tr>
<td>Bacteria in a normally sterile fluid</td>
<td></td>
</tr>
<tr>
<td>Evidence of pneumonia with purulent sputum</td>
<td></td>
</tr>
<tr>
<td>Severe Sepsis: Sepsis + Evidence of organ dysfunction</td>
<td>Cardiovascular – Use of vasopressors or systolic BP &lt;90 mmHg or MAP &lt;70 mmHg despite fluid resuscitation</td>
</tr>
<tr>
<td>Renal: urine output &lt;0.5ml/kg/hr</td>
<td></td>
</tr>
<tr>
<td>Respiratory: mechanical ventilation or PaO$_2$/FiO$_2$ ≤ 250 mmHg</td>
<td></td>
</tr>
<tr>
<td>Hematologic: platelet count &lt; 80,000 cells/mm$^3$</td>
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</table>

**Table 1-1** Criteria used to diagnose a septic patient [6, 8, 35]. This table illustrates common physiological parameters that are used to diagnose a patient as septic.
1.5  Early Goal-Directed Therapy in Sepsis

While there is difficulty in adequately defining sepsis, early recognition and associated intervention is associated with improved survivability. The ability to recognize sepsis in the early stages of the disease, when treatment would be most effective, is difficult. As sepsis develops, hypovolemia, myocardial depression, increased metabolism and loss of vasoregulation create an imbalance between oxygen supply and demand by the vital organs[36]. Organ dysfunction quickly ensues, and if unrecognized, leads to increased mortality in the early phase of septic onset[37]. Due to perturbations in systemic circulation, the body is not able to match oxygen supply to demand, and attempts to compensate by increasing oxygen extraction, which consequently leads to a decrease in mixed venous oxygen. This in turn, leads to increased lactate production as the oxygen extraction limit is reached, which is a hallmark of early sepsis. The early-phase recognition of sepsis is the primary target of early goal-directed therapies aimed at preventing the dysfunction cascade that progresses very rapidly once it is initiated.

The systemic parameters that are relied upon to diagnose sepsis in the early phase are usually CVP, MAP, and lactate levels. The monitoring of these parameters is standard practice in the clinical setting, and allows for early identification of patients at risk of becoming septic. Early goal-directed therapy involves aggressive fluid resuscitation in an attempt to increase intravascular volume, venous saturations, and to optimize systemic hemodynamic parameters (CVP, MAP)[36]. It has been shown that fluid therapy in the early phase of sepsis has a survival benefit, which is the reason that resuscitation is very common, and identification of an ideal fluid for resuscitation is important[38]. The drawback of early goal-directed therapy is that achievement of systemic targets does not directly indicate restoration of the microcirculatory environment; oxygen delivery and inflammation-mediated perfusion impairment may still persist, resulting in local tissue hypoxia.

1.5.1  Resuscitation Fluids

The primary goal of resuscitation fluids and early goal-directed therapy is to restore the intravascular volume of hypovolemic patients [39]. Choice of fluid type for resuscitation
is highly debated amongst ICU physicians; however, early administration of fluids is associated with decreased mortality[3]. Two of the most commonly used fluids for resuscitation are saline and albumin, and there have been several studies and meta-analyses (SAFE, CRYCO, Delaney (Meta-analysis), Cochrane Group (Meta-Analysis)) that have attempted to determine which resuscitation fluid best improves mortality in sepsis, to no avail[40-43]. One potential explanation of this consistent discrepancy is due to the variability in patient recruitment within these clinical trials. The presence of co-morbidities and the timing of sepsis diagnosis vary greatly in the ICU, which makes interpreting the efficacy of resuscitation fluid inherently difficult.

Mechanistically, a bolus injection of fluid into the venous system for fluid resuscitation takes advantage of the Frank-Starling mechanism, by increasing right arterial pressure, leading to increased cardiac output. The bolus injection increases venous return by increasing systemic pressure due to volume increase, which causes increased flow to the right heart. In addition, the large volumes of fluid administered in resuscitation regimens result in the hemodilution of the blood. This reduces the resistance of the blood in the circulation due to slightly decreased viscosity, which is another reason that cardiac output and venous return increase[44].

Physiologically, the main difference between albumin and saline is that albumin generates colloid osmotic pressure (COP), or oncotic pressure, which is responsible for drawing water into the vasculature. COP is inversely proportional to extravascular fluid flux, thus maintaining intravascular volume[45]. Albumin is able to maintain COP due to the Van’t Hoff’s equation:

\[
Osmotic\ Pressure\ (\pi) = \frac{RTc}{M} + kc^2
\]  

(1)

Where R is the universal gas constant, T is the temperature, M is the molecular mass of the substance, c is the concentration, and k is a constant. From this equation it can be
deduced that osmotic pressure is proportional to concentration, and inversely proportional to molecular mass. Albumin is the major contributor of oncotic pressure in the blood as it has the smallest molecular weight (69kD) of all the plasma proteins, and it is the most abundant. The osmotic effect of albumin is further increased by its negative charge, which causes attraction of cationic molecules into the intravascular environment[46].

Saline on the other hand is not able to maintain osmotic pressure, and causes dilution of the oncotic agents in the normal blood plasma. Thus saline resuscitation leads to decreased COP, causing increased filtration of water across the capillary membrane. This causes a greater increase in interstitial fluid than intravascular volume, and illustrates why higher volumes of saline would be required to achieve the same degree of intravascular volume expansion as albumin.

A comparison of physiological characteristics between 5% albumin and saline is provided (Table 1-2). In addition to osmotic pressure, albumin has a number of potentially protective roles, especially within compromised microvasculature. Albumin decreases the permeability of the vasculature, and decreases the leakage of fluid by down regulation of vascular inflammation. Though the exact mechanism is not known, the negative charge of albumin may repel membrane proteins; the size of the albumin molecules may reduce the size of endothelial pores thereby reducing vascular leak.

The protective effect of albumin is further supported by the finding that albumin plays a role in preventing endothelial cell apoptosis which, when considering the microvascular dysfunction associated with sepsis, may provide a survival benefit[47]. Albumin has anticoagulant effects similar to heparin, and acts to lessen leukocyte-endothelial cell interaction and platelet aggregation. Albumin also has binding capabilities, which allows albumin to act as a carrier for antibiotics and many other potential therapeutics[46]. Hypoalbuminemia is associated with poor prognosis, and exogenous administration of albumin has been found to produce a survival benefit in these patient cohorts. It remains unknown whether the lack of albumin in these patients causes poor outcomes, or is merely an indicator of a more severe issue[46].
The drawbacks of albumin are that it is relatively expensive, and due to the fact that it is created by pooled human plasma, has the potential to transmit infection. Furthermore, there are some cohorts of patients, such as those present with traumatic brain injury, that have significant increases in mortality with albumin resuscitation, such that albumin resuscitation should be avoided in these cases.

The drawback of saline, besides a greater tendency for tissue edema, is the potential for hyperchloremic metabolic acidosis. This is caused by an overall decrease in blood pH, due to dilution of blood bicarbonate, which would be stimulated by the large amount of saline required to achieve resuscitation targets. Hyperchloremic metabolic acidosis caused by large volumes of saline resuscitation may cause increased inflammation and mortality in sepsis[48].

Although in theory albumin appears that it would be more effective as a resuscitation fluid, high-quality evidence supporting the beneficial effect of albumin is lacking[41]. The secondary aim of this thesis was to provide insight into the efficacy of albumin versus saline as resuscitation fluids. While many different fluids are still used clinically, the decision to begin with 5% human albumin and saline as the first application of our model lies in the relevancy to global critical cares. In an animal model with a controlled insult, the administration of bolus fluids was implemented in accordance with clinical hemodynamic targets to maintain translational integrity.
### Table 1-2
Comparison of physiological characteristics of the most commonly used colloid and crystalloid fluids for resuscitation[45].

<table>
<thead>
<tr>
<th></th>
<th>Colloid</th>
<th>Crystalloid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>5% Albumin</strong></td>
<td><strong>0.9% Saline</strong></td>
</tr>
<tr>
<td>Molecular weight, Average (kD)</td>
<td>69</td>
<td>0.06</td>
</tr>
<tr>
<td>COP, mmHg</td>
<td>20-30</td>
<td>0</td>
</tr>
<tr>
<td>Maximum Volume expansion, %</td>
<td>70-100</td>
<td>20-25</td>
</tr>
<tr>
<td>Duration of Volume expansion, h</td>
<td>12-24</td>
<td>1-4</td>
</tr>
<tr>
<td>Potential for adverse reactions</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Possible Side Effects</td>
<td>Allergic Reactions</td>
<td>Hyperchloremic acidosis</td>
</tr>
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<td></td>
<td>Transmitted Infection</td>
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</table>

### 1.6 Animal Models of Sepsis

In critical care research, the importance of animal models that accurately mimic the human septic condition is integral in the development of therapeutics, for the development of sepsis staging criteria, and to broaden understanding of sepsis pathophysiology[49]. The difficulty in carrying out effective research in humans lies in the multitude of potentially confounding variables in recruited patient cohorts. The presence of comorbidities (e.g. diabetes, pneumonia, myocardial infarction), various
sources of initial infection, and concomitant therapeutic interventions, are all sources of variability that impede valid conclusions within the clinical research environment[7]. Research difficulty is further exacerbated due to the fact that early diagnosis of sepsis is based on unspecific criteria, and the progression from SIRS to sepsis can be caused by a multitude of pathophysiological pathways[50].

Although it is difficult to perfectly replicate clinical sepsis, the ability to remove some of the variability inherent to clinical human populations using an animal in a controlled laboratory environment could be a valuable translational tool. Nevertheless, there is currently a lack of clinically relevant animal models of sepsis[51]. This is a contributing factor to the failure of so many clinical trials, and is a barrier to the development of reliable therapeutics for the treatment of sepsis[7]. Animal models are necessary not only in the development of therapeutics, but also in understanding mechanistically how the therapeutics may improve patient outcomes and mortality rates [52]. There are several fundamental issues with animal models of sepsis that make translating knowledge from the bench to the bedside difficult they include: source of induced insult, lack of physiological monitoring and intensive care, timing of treatment, lack of co-morbidities, and animal type[7, 49, 51-53].

In an attempt to mimic the human septic condition, several animal models have been developed, which can be categorized based on the type of initiated insult. The most common infective agents are: exogenous administration of toxin (e.g. lipo-polysaccharide (LPS)), exogenous pathogen administration (bacteria), and iii) endogenous disturbance of protective barrier against bacteria (e.g. cecal ligation and perforation (CLP))[49]. While each model has merits in terms of understanding response to infection, the ability to mimic the hemodynamic response in septic patients is essential. The hemodynamic response occurs in two distinct phases: the early phase (increased cardiac output, low peripheral resistance) and the late phase of hemodynamic shock (decreased cardiac output, low peripheral resistance[54]. Besides the disparity in immune response, both exogenous toxemia and bacterial infection models are also unable to replicate the human hemodynamic response, which limits the clinical applicability of these models. While these methods are still commonly used, CLP is considered the gold standard, as this
source of infection is able to effectively mimic the hemodynamic conditions attributed to the human septic response[2]. A common criticism of animal models is the lack of intervention that would ensue in a patient present with the same conditions. The CLP model creates necrotic tissue, promoting abscess formation, which would necessitate excision in a human patient[49]. The formation of an abscess is a further drawback of the CLP model; an animal can stave off the infection, effectually preventing septic onset[2]. Furthermore, puncture size and amount of expressed fecal matter causes large variation in severity of the infectious stimulus between animals, which decreases the consistency necessary to draw effective conclusions from animal experimentation. These issues are exacerbated by the lack of physiological monitoring to follow the overall status of the animal, and for comparison to output from instrumentation common to ICU patients.

The lack of intensive care monitoring in animal studies is a major drawback in models of sepsis. Septic patients are instrumented such that indicators of macrovascular data (MAP, CVP, HR, Respiratory Rate) are continuously monitored, and mechanical ventilation is usually necessary[52]. The lack of instrumentation, and thus absence of physiological monitoring, is closely associated with inconsistency in interventional techniques. If physiological data is not available, both sepsis diagnosis and therapeutic interventions are not guided by the same criteria as the clinical setting[53]. This leads to arbitrary implementation of interventions, potentially before the animal would be clinically considered septic, and creates a further disconnect between the bench and the bedside.

The lack of co-morbidities in animal models is another common point of criticism. The plethora of possible comorbidities in septic patients is misrepresented in animal models, and is a very common criticism of translational research [7, 50-52]. While introducing co-morbidities into an animal model may improve clinical relevancy, the potential for confounding variables is large, and the choice of co-morbidity would still only represent a small portion of septic patients. The use of a two-hit model in theory would provide valuable information; however, a basic one-hit model needs to first be established in order to distinguish the effect of the co-morbidity on the septic response. This is also true of therapeutic interventions, higher-level instrumentation, and septic models with longer
durations (see Fig. 1-1). The primary aim of this thesis was to develop this central node model that can act as a platform for future sepsis studies.

The use of animals, such as baboons, sheep and pigs is common to sepsis research; however, the large cost of these experiments preclude their use in large numbers, which is necessary to effectively establish the potential benefit in human patients. In addition to cost, these large animals are less sensitive to bacteria than humans, thus posing a challenge to translational research. Sheep models of sepsis have illustrated similar sensitivity to humans, and replicate the hemodynamic response of sepsis; however, there have been few survival studies in sheep, which limits the effectiveness of their use in pharmacological research[51]. The use of murine models of sepsis has come into question recently due to the discovery that the difference in genomic response to inflammation is misrepresentative of the human condition[55]. Also, the small size of mice, and low blood volume (~1ml/25g mouse) makes it difficult to fully instrument a mouse to monitor global hemodynamic changes throughout sepsis, and regular blood sampling is not possible. The larger size of rats, allows for a greater degree of instrumentation, and the ability to obtain regular blood samples. Thus far the genomic response of rats to septic insult has not been refuted, and based on experimental studies, rats phenotypically mimic the hemodynamic changes associated with the human response.
Figure 1-1 The need for a basic translational animal model of sepsis. The establishment of a central node model of sepsis is very important and would form a platform that can be altered depending on desired clinical scenario, while maintaining the accuracy and integrity of clinical practice. The formulation of this node model is of fundamental importance for global critical care research.
1.6.1 Design Constraints for a New Sepsis Model

Given the rationale for a model of sepsis that is more relevant to the clinical scenario, the aspects that need to be incorporated in order to be effective relate to macrovascular measurements, a window into the microvascular environment, controlled insult, and clinical diagnostic criteria.

Instrumentation that is able to provide systemic data over the entire course of the septic response, from implementation of insult to the culmination of the experiment, is essential. Choosing an appropriately sized animal to allow for a high degree of instrumentation, with minimal invasiveness is a primary first step. The instrumentation should provide basic vital sign data (MAP, CVP, HR, and Respiratory Rate), for the duration of the experiment. Additional instrumentation will only strength the translational application of the developed model, and will allow for a more complete representation of systemic parameters, increasing the validity of experimental findings.

Next, a window into the microvascular environment is important to be able to determine the effect that macrovascular perturbations have on the systemic microcirculation. As capillaries are the primary area for gas exchange to tissue, the ability to monitor perfusion alterations over the course of a septic insult is integral to providing clinically relevant data. As qualitative measurements are relied upon clinically, the added ability to make quantitative microvascular measurements may provide mechanistic explanations for clinical observations. Furthermore, the window into the microvasculature brings a two-sided approach to septic animal research, allowing for more accurate interpretation of results.

The ability to control the insult between animals is also important to be able to replicate the septic response. A major issue in elucidating information clinically is due to the variety of patient backgrounds and differing degrees of sepsis severity. For this reason, mimicking the response between animals, and in essence, between treatment groups, allows the data extracted to be attributed to interventional therapies, and modifications to the model, rather than due to variability in the initial insult.
Lastly, and most often overlooked is the necessity to monitor the animal after the initiation of insult, and to only intervene when the animal would be clinically diagnosed as septic. This prevents the animal from being “pre-treated” before the onset of sepsis, which could greatly improve survivability, and potentiate misleading conclusions.

## 1.7 Objectives and Hypotheses

This thesis involved two main objectives:

1) To create an animal model of sepsis, incorporating the rationale for an effective translational model

With the guidance of critical care physicians we aimed to create an animal ICU, for the instrumentation of an animal that can be monitored as a human surrogate. We aimed to monitor the physiological parameters over the entire course of the septic response, and to develop an insult that would mimic the cardiovascular events of human sepsis. We also wanted to ensure that our insult was sufficient to create an obvious sepsis diagnostic window that would be related to clinical indicators of early sepsis. Lastly we wanted to incorporate the use of a microvascular window in an organ remote the site of initial inflammation in order to assess the degree of microvascular dysfunction associated with our administration of the septic insult.

2) To test the application of our model by testing the efficacy of different fluid types for clinical hemodynamic optimization, and the associated effect on the microvascular environment

We decided to test the two most common resuscitation fluids saline and albumin, due to the debate as to which fluid better restores systemic homeostasis and improves survivability. As there is not a well-defined way to measure microvascular perfusion quantitatively, mortality, and global systemic parameters are relied upon for determination of fluid efficacy. The application of our model to test this issue permits two-sided data acquisition, allowing for the characterization of fluids based not only on global hemodynamic targets, but also on the ability to prevent/restore microvascular perfusion. **Our goal was to test the efficacy of saline and albumin as resuscitation fluids**
with respect to optimization of hemodynamic parameters at the macrovascular level, and restoration of microvascular perfusion.
1.8 References


Chapter 2

Developing a translational animal model of sepsis

2.1 Introduction

Despite global research efforts aimed at improving outcomes in sepsis, the associated systemic inflammatory response and multiple organ failure remains the major cause of mortality and morbidity [1]. Although understanding of the pathophysiology of sepsis is improving, translating the pre-clinical knowledge of sepsis from the bench to the bedside remains a major challenge [2]. A recent international roundtable discussion aimed at improving clinical trials in the critically ill emphasized the need for a translational animal model of sepsis that is more representative of the clinical scenario [3]. The discussion was spawned by the failure of over 60 randomized clinical trials, citing the shortcomings of previously developed animal models as a major contributing factor. The main issue reported in this article is that many animal models fail to consider the physiological status of the animal over the entire course of a septic insult. The lack of a clinically relevant animal model of sepsis is a major obstacle in terms of developing effective therapies for sepsis [4]. The failure to use a clinical definition of sepsis to initiate potential therapies may also cause misleading conclusions, and thus ineffective translational research. In addition, the authors noted that it is important to be able to tightly control an experimental system, especially in a heterogeneous disease state such as sepsis. For this reason, it is important to choose an animal type, and a route of induced infection that will adequately and consistently mimic the human septic response [4-6].

In terms of replicating human sepsis, it is common to divide the response into two phases: early and late sepsis. Early sepsis is often characterized by an increase in cardiac output, and low peripheral resistance, whereas late sepsis involves low cardiac output, while still exhibiting low peripheral resistance [5]. The difficulty representing the early phase of sepsis in an animal model can be attributed to early goal-directed therapies in the clinical treatment of septic patients. Clinically, in the early phase of sepsis, a patient has likely already received antibiotic and fluid therapy, inducing a hyperdynamic circulatory state. As previously mentioned, in creating a clinically relevant animal model of sepsis, it is
important to illustrate the baseline effect of a septic response prior to intervention implementation. For this reason, short-term animal models of sepsis prior to any interventions are more likely to represent the transition from “pre-sepsis” to early phase sepsis, which is commonly associated with a drop in cardiac output, and an increase in peripheral resistance[7, 8]. Representing this transition phase is important in terms of elucidating the mechanistic effect of early-goal directed therapies implemented at the time of diagnosis of sepsis. Clinically, peritonitis represents 20-25% of infections leading to severe sepsis[9]. Several rat models of septic peritonitis have been established, and the response to infection is well documented and understood. For these reasons, the use of a peritonitis-based insult serves as a strong foundation for the development of a consistent and comprehensive model of clinical sepsis[7, 8, 10, 11].

The goal of our study was to create an animal model of sepsis, which involved clinical experience from ICU physicians in an effort to create a platform that promotes effective translational research. Considering recommendations from the international roundtable discussion, as well as input from the Canadian Critical Care Translational Research Group, we created a clinically relevant model that closely resembles nosocomial sepsis. We present a two-sided approach in the creation of our model in that we have the unique ability to measure both macrovascular and microvascular parameters over the entire course of a septic response. Intensivists, to ensure clinical accuracy, guided our novel approach and the associated diagnostic and instrumentation protocols. Our use of a flow probe on the abdominal aorta, as well as a pulse transducer catheter in both the carotid artery and jugular vein allow us to accurately measure CO, MAP, HR, and CVP for the entire course of our experiment. This high level of instrumentation allows us to accurately calculate peripheral resistance, and stroke volume without relying on observer tracings of aortic diameters[12].

Additionally, we use dual-wavelength intra-vital video microscopy (DλIVVM) to observe the microcirculation in the hindlimb muscle of our animals. Utilizing the differing light absorption properties of oxy- and deoxyhemoglobin we are able to elucidate the oxygen saturation changes over the course of a septic insult. This measurement technique is valuable for validation of our model as we are able to monitor macrovascular and
microvascular changes from “pre-sepsis” to early sepsis [13, 14]. A detailed diagram illustrating the parallels of our animal model, and a patient in the ICU is provided (Fig. 2-1).

This model will act as a stepping-stone for future studies, and can be easily adapted to match the ever-evolving demands of critical care research. The aim of this paper is to illustrate the capabilities of our animal model by demonstrating our instrumentation, data collection, and analysis techniques.
Figure 2-1 Parallels between instrumentation of a human patient in the ICU, and our animal model. Human heart rate is measured using an electrocardiograph; cardiac output is measured using the thermodilution technique via a Swan-Ganz catheter implanted into the pulmonary artery, microcirculation is qualitatively assessed using side-stream dark field imaging. Animal model instrumentation is described in the methods.
2.2 Materials and Methods

Animal protocols were approved by the Animal Care and Use Committee of the University of Western Ontario (see appendix A). Sprague-Dawley rats were used in this study; male rats were used exclusively in order to eliminate the effects of the estrous cycle[15].

2.2.1 Animal Instrumentation

The rats (100g on delivery) were housed in the animal quarters at the University of Western Ontario and acclimatized for 7 days after delivery. On the day of the experiment rats were randomized to FIP or sham laparotomy groups, and weighed to verify that their mass was within experimental range (170-210g). Animals were anesthetized via intraperitoneal injection of sodium pentobarbital (6.5 mg/100g body weight). A rectal thermometer was inserted to monitor temperature, and a heat lamp was used to maintain a core body temperature of 37°C. The trachea was cannulated to allow mechanical ventilation at a rate of 73-76 breaths/min with a flow-controlled air mixture of 30%O₂/70%N₂. The left common carotid artery was cannulated with polyethylene tubing (I.D. 0.58mm, O.D. 0.965mm). The carotid artery cannula allowed heart rate and mean arterial pressure to be monitored and recorded via the amplified signal of an attached pressure transducer and PowerLab system (model ML118 PowerLab Quad Bridge Amplifier; model MLT0699 BP Transducer, ADInstruments, Colorado Springs, CO, USA). The right external jugular vein was cannulated using Silastic tubing (I.D. 0.64mm, O.D. 1.19mm) with a beveled end through which saline (0.9%) was continuously infused for minimal fluid resuscitation (0.5 ml saline/h). Supplemental doses of anesthetic were delivered via the jugular catheter as needed to maintain sufficient anesthesia for the duration of the experiment (7 hours). The jugular vein cannula allowed central venous pressure to be monitored and recorded via the amplified signal of an attached pressure transducer and associated software (Digi-Med LPA-400, DMSI-200 host software, Micro-Med, Louisville, KY). A Transonic perivascular flow probe cuff (2mm, model 2PSB) was placed on the abdominal aorta between the right iliolumbar artery and the right external iliac artery. The flow probe allowed cardiac output to be continuously
monitored and recorded via the PowerLab system. The incision was then sutured closed in two layers.

The extensor digitorum longus (EDL) muscle was blunt dissected and externalized as previously described[16]. Briefly, a small section of skin was excised from the lateral side of the right hindlimb in order to expose the underlying fascia capsule. Superficial dissection of the capsule and blunt separation of the surrounding muscle allowed the EDL to be separated from the surrounding muscle tissues. Once isolated, silk ligature was threaded beneath both the intact muscle, and the attached distal portion of the EDL tendon and secured with a square knot. The tendon was severed between the knot and EDL muscle insertion, leaving the ligature securely attached to the free end of the EDL tendon. After EDL dissection, the animal was transferred to the microscope stage and placed in a supine position. The midline stitches were removed, and peritonitis was induced via intraperitoneal injection of autologous feces (FIP) (0.3 ml/100g rat of fecal slurry (1.5g feces in 1.5 ml saline)). The FIP slurry was made fresh prior to the start of instrumentation. The sham procedure consisted of removing the midline stiches, and injection of mock-FIP (0.3 ml/100g saline) into the abdominal cavity. See Appendix C.

The incision was then sutured closed in two layers and the animal placed in the right lateral decubitus position. The ligature attached to the EDL tendon was placed on the microscope stage such that the lateral side of the muscle faced the objective lenses. The ligature was then taped to maintain a length and angle approximate to the in situ position of the muscle. The muscle was moistened with 37°C saline (0.9%) and covered on the medial side with a small rectangle of plastic film (2 1 cm, polyvinylidene chloride, Saran) and a glass coverslip sealed with vacuum grease to isolate the muscle from the external environment.

2.2.2 Dual Wavelength Intra-Vital Video Microscopy

The acquisition system was composed of an Olympus IX81 inverted microscope (Olympus objective X20/0.75 numerical aperture) fitted with a 75W xenon lamp to transilluminate the muscle, a beamsplitter (Photometrics-DC2) with a filter cube containing interference filters at wavelengths 442-nm and 454-nm, and two Q-Imaging
Rolera-XR digital video cameras attached to the beamsplitter. Adjustments on the beamsplitter made it possible to register the two images and make them parfocal for simultaneous video capture at the two wavelengths. After allowing the muscle to equilibrate (30 minutes) real-time microvascular blood flow video sequences (696 X 520, 21 frames/second) were taken of in-focus capillaries within the EDL muscle. The videos captured at the wavelengths (442-nm and 454-nm) were chosen to utilize the unique light-absorption properties of oxy- and deoxyhemoglobin similar to the previously described system[17]. The 454-nm wavelength is an isosbestic wavelength, whereas absorption at the 442-nm wavelength changes with oxygen saturation.

2.3 Experimental Protocol

2.3.1 Video Recordings

After the intraperitoneal injection (baseline), the physiological status of the animal was continuously monitored, as previously outlined. Microvascular blood flow video sequences were taken of in-focus capillaries in 10 distinct fields of view. Recordings were made at baseline (after equilibration), 3 hours, and 5 hours post-injection.

2.3.2 Blood Samples

After the video sequences were recorded 250μl of heparinized arterial blood was collected (baseline, 3, 5 hours). Once the sample was collected the blood volume was replaced with heparinized saline. To measure systemic blood variables, an iSTAT point-of-care system was used, along with associated cartridges (CG4+: Lactate, blood gases, CG8+: Na, K, iCa, Gluc, Hct, blood gases).

A timeline representation of the above methodology is provided (Fig. 2-2).
Figure 2-2 Timeline of study for animal model. Blue arrows indicate the timing of video captures, as well as timing of blood collection. Red asterisk indicates administration of FIP, and BL indicates the timing of baseline measurement collection. The animal is minimally resuscitated with an infusion of saline (0.9%, 0.5 ml/hr). MAP, HR, CVP, and CO are measured for the entire observation period.

2.3.3 Video Processing and Functional Images

At the culmination of the experiment, the recorded video sequences were processed and stored as uncompressed 16-bit PNG files using custom capture software (Neovision). The video sequences from both wavelengths were processed using Matlab algorithms, as previously described, which produce functional images[18]. These images allowed delineation of hemodynamic and O₂ saturation measurements for individual capillaries, using custom software developed in our lab. Briefly, the functional images allow the software to accurately determine the coordinates of the lumen of the selected capillaries, as well as the centerline of the vessel. Once the vessel is traced, light intensity levels along the centerline of the vessel are extracted over the sample interval producing space-time images (STIs), one for each wavelength. Measurement of average velocity, O₂ saturation, RBC supply rate, hematocrit, and diameter were made from the analysis of the
STIs. All in-focus vessels, from each field of view, at each timepoint, were analyzed using the same technique. A total of 16 in focus capillaries were analyzed in the hindlimb muscle of the presented animal. See Appendix B.

2.3.4 Functional Capillary Density Measurement

Capillary densities were determined from the captured video sequences by counting the number of capillaries that crossed three staggered overlaid test lines (200μm each) perpendicular to the direction of the EDL muscle fibers as described previously[14]. A total of three staggered test lines were overlaid for each recorded field perpendicular to the direction of the EDL muscle fibers. The capillaries that intersected the test line were categorized over a 30 second interval based on the degree of RBC transit as either: continuous, intermittent, or stopped. A capillary was considered continuous if the flow was uninterrupted over the sequence, intermittent if flow had at least 3 seconds of stopped flow, or had a period of reversed flow direction, or stopped if the capillaries did not have any flow over the course of the recorded video sequence. Capillaries that did not have RBC’s were not included in capillary density measurement. Capillary density was counted for each field of view, at each time point, and averaged to determine the change in capillary density over the course of the septic response. In the presented data, 10 fields of view were measured to determine functional capillary density.

2.3.5 Physiological Data Analysis

The physiological data collected for the duration of the experiment were analyzed using LabChart software. A moving average was used to analyze the collected variable signals (MAP, CVP, CO, HR). A 300 second (5 minute) averaging window was chosen, and the average of each interval was taken over the course of the experiment (5 Hours). This was done to eliminate major deviations within the signal in order to extract the physiological effect of the insult alone. Stroke volume (CO x HR) and total peripheral resistance (MAP-CVP/CO) were calculated at the culmination of the experiment.
2.3.6 Physiological Variables

The clinical adaptability of our model lies in the parameters we can continuously monitor. Comparing the microvascular data to the macrovascular data facilitates a better understanding of the underlying pathophysiology of the septic response.

2.3.7 Statistical Analysis

Microvascular data and functional capillary density measurements are presented as mean and standard deviation. The data presented are for one (n=1) demonstration animal, thus analysis for statistical significance was not performed.

2.4 Results

2.4.1 Animals

Examples of arterial blood measurements from a single septic animal demonstrating the global measurements collected over the course of a septic insult are found in Table 2-1. The systemic parameters were measured at 3 time points to track the progression over the course of the experiment.
<table>
<thead>
<tr>
<th></th>
<th>Hct (%)</th>
<th>Lactate (mmol/L)</th>
<th>Glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>0.43</td>
<td>1.51</td>
<td>10.3</td>
</tr>
<tr>
<td><strong>3 Hours</strong></td>
<td>0.45</td>
<td>1.06</td>
<td>9.4</td>
</tr>
<tr>
<td><strong>5 Hours</strong></td>
<td>0.46</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>pH</strong></td>
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<td></td>
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</tr>
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</tr>
<tr>
<td><strong>pO2 (mmHg)</strong></td>
<td>128</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1 Systemic variables at baseline, 3 hours, and 5 hours. All measurements were made from heparinized arterial blood collection, immediately following video capture.
Figure 2-3 Macrovascular measurements from baseline to 5 hours post insult of (A) Heart Rate (B) MAP (C) Cardiac Output (D) CVP, (E) Stroke Volume* and (F) Total Peripheral Resistance* (n=1). The superimposed line on the blood pressure graph indicates clinical sepsis inclusion criteria (<75mmHg). See Appendix C. In this example, our diagnosis of sepsis occurs around the 3.5-hour timepoint. * = Calculated
Figure 2-4 Microvascular quantification of capillary A) Oxygen Saturation, B) Hematocrit, C) Velocity and D) Supply rate (n=16 capillaries).
Figure 2-5 Functional capillary density for baseline, 3-hour and 5-hour time points to quantitatively measure the degree of microvascular dysfunction. Analyzed capillaries are categorized as either continuous flow, intermittent flow, or stopped flow capillaries based on degree of RBC transit. Values correspond to the number of capillaries in each category that cross the 600-µm test line. (n=10 fields of view)
2.5 Discussion

This study presents a novel model of sepsis that is more clinically relevant, as the high degree of instrumentation provides continuous macrovascular data, while concurrently providing a window of the microvasculature to assess the degree of dysfunction. Furthermore, our use of FIP to induce sepsis allowed us to obtain systemic measurements immediately following the initiation of insult (true baseline). The success of translating animal studies to the bedside depends on the ability to effectively document the severity of sepsis at the time of intervention. One of the main pitfalls of translational animal research is the inability to monitor the septic response over the entire course of the experiment. Thus our dual-sided approach addresses the major drawbacks of previous animal models, and will ultimately provide insight into the mechanistic effects of interventional therapies.

Clinically the efficacy of a treatment intervention will be affected by the severity of the insult. Thus the information provided by the microcirculation is integral to determining the severity of sepsis as it relates to the severity of dysfunction of the vital organs. Currently, the sublingual microcirculation is the only easily accessible clinical microcirculation that is used in an attempt to quantify changes in the microcirculation in response to sepsis. While this technique provides qualitative information regarding microcirculatory dysfunction, due to inherent technical limitations it is not currently possible to make oxygen saturation measurements, and thus provides little insight into the degree of oxygen delivery to tissues. For this reason, the microcirculatory window in our model provides valuable information that allows determination of indices of oxygen delivery to the tissues. It is our hypothesis that severity of organ dysfunction of sepsis relates to the inability to deliver adequate oxygen to the organ tissues as a result of microcirculatory dysfunction.

In terms of the recommendations from the roundtable discussion, the challenge of animal models lies in the difficulty in representing the complexity of clinical sepsis, alluding to the absence of models that focus on co-morbidities. Our model is a fundamental first step towards addressing this problem. It is of critical importance when developing a model of
human disease to have control over the entire protocol. The establishment of a reliable foundation increases the adaptability of the model, and provides a workable baseline such that the effect of modifications will be easy to detect. For example, with our established model, using an aged animal would not require any modifications to our protocol, and the effect of age on macrovascular and microvascular response to sepsis can be clearly defined. In addition, our model does represent a simple two-hit model, as the surgical procedure in instrumenting the animal requires invasive surgery. The surgical aspect of our protocol, however, is minimal and therefore more predictable in terms of inflammatory response of the animal (due to surgery). Taken together with the addition of a septic insult, our model effectively represents clinical nosocomial sepsis arising secondary to an initial trauma.

Utilizing FIP to induce sepsis also has distinct advantages over the use of endotoxin administration or cecal ligation methods. Firstly, although CLP is considered the “gold standard” in sepsis research, the procedure still introduces an inflammatory response within the peritoneum, which can be variable depending upon the degree of fecal contamination at the time of surgery, and whether or not abscess formation ensues [19]. The surgical process of ligating the cecum also takes considerable time, and delays data acquisition to the extent that “baseline” measurements may be obtained up to an hour after the initial insult (see Appendix C). Further, the inflammatory responses to intra-abdominal abscess may also be variable which is dependent upon ability to contain the infected area. While the size or number of punctures can somewhat control animal mortality, the variability of fecal contamination is the major source of variability in the severity of the insult between animals[5]. The use of the FIP technique introduces a higher level of control, as the dose can be adjusted depending on the desired severity of insult. In addition, the use of autologous fecal matter provides a similar type of insult to that of a CLP without the creation of an additional inflammatory response from the surgical intervention. Furthermore, it also mimics a clinical scenario of fecal contamination of the peritoneum following gut perforation.

Another recommendation of the roundtable is that animal models provide insight into the mechanism of action of targeted therapeutics. In order to better predict the response of
septic patient to therapies, *in vivo* demonstration of physiological responses to an intervention is imperative. Once again our model is able to successfully address this concern due to our two-sided approach and the introduction of a true baseline in a septic animal model. As therapeutics are often given based on a patient fulfilling distinct inclusion criteria, the degree of instrumentation in our model would allow us to treat our septic animals in the same fashion. The development of our model was driven by ICU physicians in order to ensure that the future applications of this model to treatment strategies have significant clinical representation. Besides the macrovascular data, the concomitant microvascular information our model provides is of utmost importance. While many clinical therapeutics target macrovascular endpoints, knowing the associated effect on the microvasculature can provide valuable mechanistic information. For example, fluid resuscitation strategies generally target MAP as an endpoint; however, it is still unknown what macrovascular targets are the best predictors of improving oxygen delivery to the microcirculation and if implementing microvascular endpoints of various therapies may augment survivability. The demonstration animal in this study was minimally resuscitated (0.5 ml/saline/hr) in order to illustrate the macrovascular effect of the septic insult exclusively without the addition of fluid therapy.

The absence of continuous flow vessels at the 5-hour time point in figure 2-5 does not affect our ability to make velocity and oxygen saturation measurements post experiment. The processing software only requires that there is flow in the vessel at some point during the video capture sequence. For this reason data can be extracted from intermittent flow vessels even though they may have periods of stopped or reversed flow.

As closely as we have represented the clinical scenario, however, there are limitations and potential additions that could improve the integrity of our model. In this model, we rely upon MAP to indicate septic onset, as we already have a documented source of infection; however, there are several other metrics utilized clinically that we are unable to measure in our model. Firstly, urine output is commonly used to assess adequacy of fluid resuscitation and to some degree, kidney dysfunction, which is a common indicator of early sepsis. While it is possible to catheterize a rat, the technique is far more invasive and complex than the same process in a human patient. Furthermore, the use of
pentobarbital anesthetic suppresses the central nervous system, preventing passive urinary flow. Due to the already invasive nature of our protocol, we chose not to catheterize the bladder; however we could adapt our protocol to include this measurement in the future. See Appendix C. Additional criteria that are used in diagnosis of clinical sepsis include heart rate, and timing before requirement of mechanical ventilation. Our choice of pentobarbital anesthetic requires mechanical ventilation early on in the surgical procedure, prior to administration of the septic insult. While other anesthetics more readily promote natural breathing, which would allow us to include time until mechanical ventilation requirement, pentobarbital has the least interfering effect on the cardiovascular parameters that are a strength of our model[20]. Furthermore, heart rate > 90 bpm is used clinically as an indicator of early sepsis. It is difficult to use rat heart rate as a reliable indicator of sepsis as the heart rate of a rat is 6 to 7 times greater than that of a human, and there is a large amount of innate variability in the heart rate signal. For this reason we do not use heart rate as an indicator of systemic sepsis. Next, we present an acute model of sepsis in that the septic response we instigate is monitored for 5 hours. While the ability to completely mimic a human septic response would last several days, our microcirculatory preparation is terminal. In order to completely duplicate the timing of a human response we would have to sacrifice our microcirculatory data collection, which is a pivotal advantage our model has over previous animal models.

In terms of adding to our model, harvesting the vital organs at the culmination of our experiment could identify markers and pathological injury of sepsis, and would act to bolster the relevance of our model to the clinical scenario. Next, measuring white blood cell count, platelet, and creatinine levels would be a simple addition that would improve the clinical relevancy of our model. Lastly, utilizing other techniques, such as near-infrared spectroscopy, to assess the microvascular environment surrounding easily accessible vital organs (brain, kidney etc.), would reinforce our use of the EDL as representative vital organ vasculature.

In summary, our model presents a valuable tool in translational research, with an abundance of potential applications. The model has many moving parts that can easily be modified to meet the demands of critical care, and sepsis researchers alike. The model
also serves as a valuable learning tool for clinicians with our unique ability to illustrate the systemic affect of therapeutic interventions.

2.6 Conclusion

The presented model achieved our goal of creating a translational animal model, guided by ICU physicians, that is representative of a clinical septic response. In addressing the recommendations of the international roundtable discussion on animal research in sepsis, we created a well-defined, repeatable model with a high level of control over the severity of insult. This model lays the groundwork for future studies aimed at elucidating the effects of interventional techniques such as fluid resuscitation, and impact of early goal-directed therapies on the microvascular environment.
2.7 References


Chapter 3

Assessing fluid resuscitation efficacy in a translational animal model of sepsis

3.1 Introduction

The systemic inflammation induced by sepsis poses significant challenges to the circulatory system and causes local regions of hypoxia in the vital organs[1]. The fast progression of this disease is associated with the imbalance between oxygen delivery and oxygen demand, which leads to multiple organ failure, and high mortality rates in the ICU[2]. The imbalance is due to a multitude of circulatory effects including hypovolemia, myocardial depression, peripheral vasodilation, and increased tissue metabolism[3]. It is well established that early recognition of sepsis leads to better clinical outcomes, and improved survivability[4]. Early goal-directed therapy guided by physiological targets is fundamental to improving patient outcomes, and administration of fluids for resuscitation is often the first strategy to be implemented[3]. As of yet, there is no “gold standard” fluid used for resuscitation, and there is constant debate among clinicians as to which type of fluid best restores macrovascular and microvascular targets[5]. Saline and albumin, two of the most commonly used resuscitation fluids, are at the center of this debate. Several clinical trials and meta-analyses have attempted to demonstrate conclusive evidence of an ideal resuscitation fluid; however, the findings, much like the opinions of clinicians, were mixed[6-8]. While clinical trials and meta-analyses are able to involve large cohorts of patients, there are several sources of variability inherent to patient recruitment that make it difficult to draw definitive conclusions. Variability in patient background, concomitant therapies, timing of sepsis diagnosis, and timing of intervention, all contribute to these discrepancies[9]. For this reason, elucidating the actual mechanistic effect of fluid type on resuscitation is intrinsically difficult. Furthermore, although fluid resuscitation strategies often target global hemodynamic parameters, the mortality rate of sepsis remains relatively stagnant. Recently, it has been shown that the macrocirculation and the microcirculation may be differentially regulated in sepsis, which further demonstrates the need for considering the effect of early goal-directed therapies on the microcirculatory environment[10-12]. The
lack of evidence of fluid efficacy using translational animal models of sepsis only exacerbates these pitfalls, defining a clear disconnect between the bench and the bedside.

Using our previously developed animal model of sepsis we are able to test the effect of fluid administration on both the macrovascular and microvascular environment, over the entire course of a septic response. In active collaboration with the Canadian Critical Care Translational Research Group, and awareness of the PRECISE study, we decided to test the effect of the most common resuscitation fluids: 0.9% saline, and 5% albumin[13]. There are currently no clinical guidelines that determine the use of one resuscitation fluid over the other, thus comparing the efficacy of each fluid is a reasonable first application of our model.

Saline is the most commonly used resuscitation fluid. Its purpose is to increase or maintain vascular volume, however saline does not have the ability to maintain osmotic pressure, and readily leaks into the extravascular environment. For this reason, a higher volume of saline is generally required to maintain hemodynamic optimization[14].

We used 5% human albumin in accordance with common clinical concentrations. Albumin is used as a resuscitation fluid since it maintains colloid osmotic pressure (COP), which limits extravascular leakage[15]. Albumin also has anti-inflammatory properties, prevents platelet aggregation, and may play a role in maintenance of endothelial cell function[16].

The goal of this study was to utilize clinically relevant hemodynamic optimization targets, in conjunction with our previously developed translational animal model of sepsis, to determine the macrovascular and microvascular consequence of saline and albumin as resuscitation fluids. We utilize dual wavelength intra-vital video microscopy (DλIVVM) to provide a window into the microvasculature, in an organ remote to the site of septic insult, that allows us to monitor the progression of microvascular dysfunction at the onset of sepsis. This gives us the unique ability, in conjunction with a fully instrumented animal, to provide physiological data similar to a patient in the ICU, with the addition of a quantitative measurement of microvascular perfusion and oxygen transport. While the sublingual microcirculation is relied upon clinically to assess
microvascular perfusion, the measurement process is semi-quantitative at best and is subject to many shortcomings that make bedside assessment in a critical care environment fundamentally difficult[17]. Our novel approach provides accurate measurements of oxygen saturation, velocity, supply rate, and hematocrit of RBCs passing through single capillaries in the microvascular network. While we are able to utilize scoring criteria to provide data similar to sublingual measurements, incorporating RBC saturations into our sepsis model elucidates a true mechanistic effect of fluid administration on tissue oxygenation, which greatly increases the translational ability of our approach.

This paper presents preliminary results of our study investigating the effect of fluid type on optimizing clinical hemodynamic targets, while concurrently providing insight into the restorative ability of each fluid on the degree of microvascular dysfunction. We report for the first time that after induction of sepsis, using a MAP of 75mmHg as our resuscitation target, bolus injections of 5% albumin appear to better restore macrovascular and microvascular parameters, compared to saline. Despite the ability to restore MAP, saline had little effect on both cardiac output and microcirculatory perfusion, with endpoints similar to our septic control animals. These preliminary data suggest that albumin, administered at the onset of sepsis, is a more effective resuscitation fluid in terms of hemodynamic optimization and, in-line with the emerging clinical goal of microvascular restoration, may provide a protective effect in reducing the hypoxic environment in the vital organs.

### 3.2 Methods

Animal protocols were approved by the Animal Care and Use Committee of the University of Western Ontario (see appendix A). Sprague-Dawley rats were used in this study; male rats were used exclusively in order to eliminate the effects of the estrous cycle[18].

A timeline of the experimental protocol is provided (Fig. 3-1).
3.2.1 Feces Induced Peritonitis (FIP)

Peritonitis was induced via intraperitoneal injection of autologous feces (FIP) (0.3 ml/100g rat of fecal slurry (1.5g feces in 1.5 ml saline)). The fecal slurry was made on the day of the experiment. Sham animals received saline (0.3ml/100g rat, 0.9% saline) as opposed to FIP slurry.

3.2.2 Animal Instrumentation

The rats (100g on delivery) were housed in the animal quarters at the University of Western Ontario and acclimatized for 7 days after delivery. For these experiments we used our previously developed translational animal model of sepsis. Briefly, on the day of the experiment, rats were randomized to FIP, FIP-Saline resuscitated (0.9% Saline, B.Braun Medical Inc., Irving, CA), FIP-Albumin resuscitated (Alburex 5, CSL Behring, Ottawa, ON), or sham laparotomy groups, and weighed to verify that their mass was within experimental range (170-210g). Animals were anesthetized via intraperitoneal injection of sodium pentobarbital (6.5 mg/100g body weight), and mechanically ventilated at a rate of 73-76 breaths/min with a flow-controlled air mixture of 30%O₂/70%N₂. Temperature was monitored using a rectal probe, and maintained at 37°C. The right carotid artery, and left jugular vein were cannulated to monitor heart rate and MAP, and CVP respectively, via attached pulse transducers. Supplementary anesthetic, and saline were administered via the jugular catheter, as needed. The bolus injection of fluids was also administered via the jugular catheter, as required to maintain clinical optimization targets. Cardiac output was measured using a transonic flow probe attached to the abdominal aorta between the right iliolumbar artery and the right external iliac artery. The incision was then sutured closed in two layers.

The extensor digitorum longus (EDL) muscle was blunt dissected as previously described[19], and reflected onto the stage of a dual-wavelength intra vital video microscope. Midline stitches were then removed and peritonitis was induced with an intraperitoneal FIP injection. The sham animals received the same volume of saline. The muscle was then secured via attached silk ligature at its in situ length, and relative orientation, and was isolated from room air using a small rectangle of plastic film (2 x 1
cm, polyvinylidene chloride, Saran) The muscle was moistened with 37°C saline (0.9%) and a glass coverslip sealed with vacuum grease to isolate the muscle from the external environment.

### 3.2.3 Dual Wavelength Intra-Vital Video Microscopy

The acquisition system was an inverted microscope fitted with a xenon lamp for transillumination. A beam splitter with a filter cube containing interference filters at wavelengths of 442-nm and 454-nm, which allowed for dual-wavelength video capture. After allowing the muscle to equilibrate (30 minutes) real-time microvascular blood flow video sequences (696 X 520, 21 frames/second) were taken of in-focus capillaries within the EDL muscle. Post-processing using the dual wavelength system permitted calculation of RBC velocity, supply rate, hematocrit, hematocrit, and oxygen saturations, of the in-focus capillaries within the EDL muscle as previously described [20, 21].

### 3.2.4 Clinical Hemodynamic Optimization Target

The hemodynamic optimization target that we used for our study was motivated by clinical inclusion criteria provided by our collaborative knowledge users. The criteria we used in our protocol to begin resuscitation was a MAP <75mmHg. Once the MAP of the animal dropped below this threshold 1ml bolus injections of the resuscitation fluid were administered into the venous system. This process was repeated as necessary to maintain MAP > 75mmHg. Consecutive bolus injections were often needed, however, a 5-minute window between injections was used in order to prevent over-resuscitation due to response from the previously injected bolus. Bolus injections were never given during the recording of the 3-hour videos, to prevent misrepresentation in post-processed capillary measurements.

### 3.3 Experimental Protocol

#### 3.3.1 Video Recordings

After intraperitoneal injection of the FIP (baseline), the physiological status of the animal was continuously monitored, as previously outlined. Microvascular blood flow video sequences were taken of in-focus capillaries in 10 distinct fields of view (FOV) (one
minute recording for each FOV). Recordings were made at baseline (after equilibration), 3 hours, and 5 hours post-injection.

### 3.3.2 Blood Samples

After the video sequences were recorded 250 μl of heparinized arterial blood was collected (baseline, 3, 5 hours). Once the sample was collected the blood volume was replaced with heparinized saline. To measure systemic blood variables, an iSTAT point-of-care system was used, along with associated cartridges (CG4+: Lactate, blood gases, CG8+: Na, K, iCa, Gluc, Hct, blood gases).

### 3.3.3 Capillary Analysis

Measurement of average velocity, O₂ saturation, RBC supply rate, and hematocrit were made from the analysis of the post-processed video capture sequences. All in-focus vessels, from each field of view, at each time point, were analyzed using the same technique. See Appendix B. The capillary bed within the muscle is inherently heterogeneous in terms of microvascular geometry, blood flow and oxygen transport. We attempted to obtain a representative sample by randomly selecting capillaries in 10 distinct FOV. It is expected that the impact of sepsis or resuscitation would uniformly affect microvascular perfusion, so these data are presented as percent change from baseline, as it is more representative of the microvascular alteration over the course of the experiment.

### 3.3.4 Functional Capillary Density Measurement

Capillary densities were determined from the captured video sequences by counting the number of capillaries that crossed three staggered overlaid test lines (200 μm each) perpendicular to the direction of the EDL muscle fibers as described previously[22]. Capillary density was counted for each field of view, at each time point, and averaged to determine the change in capillary density over the course of the septic response.
3.3.5 Physiological Data Analysis

The physiological data collected for the duration of the experiment was analyzed using LabChart software. A moving average was used to analyze the collected variable signals (MAP, CVP, CO, HR). A 300 second (5 minute) averaging window was chosen, and the average of each interval was taken over the course of the experiment (5 hours). This was done to eliminate major deviations within the signal in order to extract the physiological effect of the insult alone. Stroke volume (CO x HR) and total peripheral resistance (MAP-CVP/CO) were calculated at the culmination of the experiment. The positioning of the cardiac output probe on the abdominal aorta is subject to variability between animals, due to influence of the anesthetic, and the fact that the volume flow measured at this anatomical position does not include flow to the brain, brachial arteries, kidneys liver or the splanchnic organs. For this reason, percent change in cardiac output was used, as it is more representative of the changes that occur over the course of the septic response. For this reason stroke volume and total peripheral resistance are also presented as percent change, as these calculations are reliant upon cardiac output data.

3.3.6 Statistical Analysis

The time plots of the physiological data were not analyzed statistically, and are provided for qualitative purposes. In order to analyze the physiological data, box-and-whisker plots were made for the 3 time point blocks (baseline, 3 hours, 5 hours) and are presented as median and inter-quartile range (IQR). Continuous variable differences between the 4 treatment groups at the 5-hour timepoint were compared using the Kruskal-Wallis test, followed by post-hoc analysis via Bonferri-Dunn test for multiple comparisons. The capillary data are presented as mean ± standard error of the mean (SEM). These data were analyzed by one-way analysis of variance (ANOVA) with post-hoc Bonferri-Dunn test for multiple comparisons. A p-value of 0.05 was considered significant. For simplicity, significance is presented for the 5-hour time point only.
3.4 Results

The global variables (Table 3-1) were not significant; however the FIP and FIP+Saline groups levels are indicative of hyperlactatemia. The FIP+Saline and FIP+Albumin groups had lower hematocrit, and lower PaO₂.

Although this is a preliminary study (n=3 per group) the administration of the different fluids caused differences in terms of macrovascular and microvascular response. Figure 3-2 shows the average physiological parameters for each group over the entire course of...
the experiment. Individual animals in all FIP groups dropped below the MAP threshold at some point during the experiment. There was a significant difference in MAP (Fig. 3-3A) at 5 hours for the FIP and FIP+saline groups compared to sham-operated control. All of the septic animals had decreased MAP compared to baseline. For CVP, FIP+Albumin group was significantly decreased (Fig. 3-3B) as compared to the sham-operated animal at 5 hours. The FIP animal had significantly decreased heart rate from baseline (Fig. 3-3C), and as compared to 5-hour sham animals. Both FIP and FIP+Saline groups had significantly decreased cardiac output (Fig. 3-3D) compared to baseline. The FIP and FIP+Saline groups had significantly increased TPR (Fig. 3-3E) compared to both the 5-hour sham, and 5-hour baseline measurements, while the FIP+Albumin group had significant decreased TPR compared to baseline. Lastly, the FIP and FIP+Saline groups had significantly decreased stroke volume (Fig. 3-3F) compared to baseline, and the FIP group also was significantly decreased compared to the 5-hour sham. None of the data for the raw microvascular data was significant.

The only data that was significant for the normalized microvascular analysis was a significant decrease in stroke volume for the FIP+Saline group compared to baseline (Fig. 3-5D). Qualitatively, for the velocity (Fig. 3-5A), oxygen saturation (Fig. 3-5B) and stroke volume (Fig. 4D) the FIP and FIP+Saline groups follow a similar trend, declining in a linear fashion throughout the course of the experiment. The FIP+Albumin group followed a similar trend in these measurements from baseline to 3 hours; however, in all 3 measurements, the trend reversed and increased from 3 hours to 5 hours. In terms of hematocrit (Fig. 3-5C) the FIP and FIP+Albumin groups increased from baseline, whereas the FIP+Saline group decreased from baseline. Similarly, there was no significant change in the functional capillary density data.
<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>FIP</th>
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<th>FIP + Albumin</th>
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</tr>
<tr>
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<td>0.375</td>
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<td>0.26</td>
<td>0.34</td>
</tr>
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<td>Additional Fluid for</td>
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<td>N/A</td>
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<td>3</td>
</tr>
<tr>
<td>Resuscitation (ml)</td>
<td></td>
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</table>

**Table 3-1** Arterial blood measurements (via iSTAT) for Sham, FIP, FIP+Saline and FIP+Albumin groups. Data shown are averages of 2 animals in each group, 5 hours after septic insult. Average fluid required to achieve physiological target are also shown where appropriate.
Figure 3-2 Physiological parameters of sham-operated, septic (FIP), septic (FIP-Saline resuscitated), septic (FIP-Albumin resuscitated) rats (n=3). (A) Mean arterial pressure, (B) Central Venous Pressure, (C) Heart Rate, (D) Cardiac Output, (E) Total Peripheral Resistance (F) Stroke Volume. The mean arterial pressure graph has a superimposed line (75mmHg) illustrating our hemodynamic optimization target.
Figure 3-3 Physiological parameters of sham-operated, septic (FIP), septic (FIP-Saline resuscitated), septic (FIP-Albumin resuscitated) rats (n=3). (A) Mean arterial pressure, (B) Central Venous Pressure, (C) Heart Rate, (D) Cardiac Output, (E) Total Peripheral Resistance (F) Stroke Volume are displayed for each time point (baseline, 3 hours, 5 hours) as median (IQR). *Difference at 5 hours between septic animals and sham (P <0.05). #Difference within group between 5 hour and baseline measurement (P<0.05).
Figure 3-4 Microvascular parameters of sham-operated, septic (FIP), septic (FIP-Saline resuscitated), septic (FIP-Albumin resuscitated) rats (n=3). (A) Velocity (B) Oxygen Saturation (C) Hematocrit and (D) Supply Rate are displayed for each timepoint as mean and individual animal values.
Figure 3-5 Microvascular parameters (normalized to baseline) of sham-operated, septic (FIP), septic (FIP-Saline resuscitated), septic (FIP-Albumin resuscitated) rats (n=3). (A) Velocity (B) Oxygen Saturation (C) Hematocrit and (D) Supply Rate are displayed for each timepoint as mean and individual animal values. #Difference within group between 5 hour and baseline measurement (P<0.05).
Figure 3-6 Functional capillary density measurements of sham-operated, septic (FIP), septic (FIP-Saline resuscitated), septic (FIP-Albumin resuscitated) rats (n=3). (A) baseline (B) 3 hour and (C) 5 hour functional capillary densities are shown. Capillaries in each field of view are categorized as continuous, intermittent, or stopped depending on degree of RBC transit. Data are presented as mean ± SEM.
3.5 Discussion

The debate as to which fluid is best used for the resuscitation of septic patients to achieve macrovascular optimization has not been resolved[6, 8, 14]. It is well known that early fluid resuscitation of septic patients improves survivability; however the effect of administered fluid on the microvascular environment is rarely considered[2]. It has been shown that microcirculatory dysfunction is a major contributor to global tissue hypoxia, which eventually leads to multiple organ failure[1]. Thus, the ability to test common clinical therapies aimed at optimizing macrovascular hemodynamic targets, and elucidate the associated effect on the microcirculation could provide valuable information with important clinical implications. In this preliminary study, we demonstrated the unique ability to test the effect of fluid resuscitation on the microvascular environment in a translational animal model of sepsis.

While the data presented are preliminary, the trends in the macro- and the microcirculatory environments are encouraging. With our developed translational animal model, we are able to consistently replicate the transition from pre-sepsis to sepsis. See Appendix C. The FIP animals all exhibited the systemic changes commonly associated with an early septic response (drop in cardiac output, increased total peripheral resistance) (Figs. 3-2, 3-3). Interestingly, the administration of bolus albumin was able to decrease these trends and had an increased cardiac output, and decreased total peripheral resistance compared to the 3-hour time point. Albumin resuscitation was also better able to maintain blood pressure about 75mmHg, evidenced by the amount of bolus injections administered, compared to saline. This makes sense given the properties of albumin, and the ability to maintain colloid osmotic pressure. In terms of stroke volume, the FIP, and FIP+Saline groups decreased from the onset of sepsis, while the FIP+Albumin group followed a similar trend at the 3-hour time point; however, much like cardiac output and total peripheral resistance, the trend reversed at the 5-hour time point, leading to an increased stroke volume. Taken together, it appears that at the macrovascular level, albumin is able to better restore hemodynamic parameters.
The trends at the microcirculatory level are similar (Figs. 3-5). In terms of velocity, oxygen saturation, and supply rate in the capillaries, both the FIP and FIP+Saline groups decline in a linear fashion after initiation of the sepsis. The albumin-resuscitated group similarly declined from baseline; however after the 3-hour time point, and the associated administration of bolus albumin injections, the trend reversed, and all three parameters increased. The hematocrit increased in the FIP and albumin groups, and decreased in the saline group. For the FIP group this could be due to vascular leak, which decreases the plasma volume. For the FIP+Albumin group, the increase in hematocrit could be attributed to the increased velocity and supply rate.

The trends in functional capillary density (Fig. 3-6) were that in the FIP and FIP+Saline groups, there was an increase in intermittent flow capillaries over the course of the experiment and a related decrease in continuous flow capillaries. The FIP+Albumin group had an increase in intermittent flow capillaries, but reversed this trend at the 5-hour time point. These trends are indicative of capillary functionality, as an increase in stopped flow capillaries has previously been shown to increase in sepsis[22]. While there was not an increase in the amount of stopped flow capillaries for the septic animals, the increase in intermittent flow is potentially an early indicator of microvascular dysfunction. In order to be able to analyze capillaries quantitatively, there needs to be RBC transit. While an increase in the amount of intraperitoneal FIP administered would cause a more severe septic response, which would promote an increase in stopped flow capillaries, we would have to lose vessels for our hemodynamic and O₂ measurements, which are a pivotal advantage of our animal model.

In terms of clinical inclusion criteria, we used a mean arterial pressure of 75mmHg as our resuscitation target, which is only one part of sepsis inclusion criteria. Besides mean arterial pressure, temperature >38°C or <36°C, respiratory rate >20 breaths/minute or PaCO₂ <32 mmHg, heart rate>90 beats/min, and WBC count ≥12,000 cells/mm³ or <4,000 cells/mm³ are also considered indicators of sepsis. Due to the use of pentobarbital anesthetic we are unable to use time until respirator requirement as a metric of sepsis onset, and the respiratory rate is controlled throughout the experiment. We are able to take blood samples to measure PaCO₂; however, the sample requires 250µl blood draws,
making regular sampling over the course of the experiment subject to hemodilution. This is the same issue with measuring WBC count regularly. We also maintain internal temperature of 37°C over the course of the experiment. In addition, it is clinically common to measure mixed venous oxygen saturation, which is difficult in our model due to the positioning of the jugular vein catheter. Unlike the carotid artery, blood does not passively flow out of the jugular catheter, thus measurement requires drawing the blood with a syringe. The clotting nature of blood requires this process to be done very quickly, and if the blood is not totally flushed from the line, will risk forfeiting CVP monitoring for the rest of the experiment. For these reasons our use of MBP < 75mmHg made the most sense given our instrumentation, and the physiological similarity in values as compared to a human (Appendix C).

Although more experimentation with these fluids needs to be done in order to truly show fluid resuscitation efficacy, it appears that albumin is a more effective fluid at restoring both microvascular and macrovascular targets. Interestingly, although saline was able to restore the blood pressure values, cardiac output, and microvascular parameters continued to decline, which supports the notion that hemodynamic optimization is not reflective of the microvascular environment surrounding in vital organs. Furthermore, the successful application of this model and the promising ability of deducing mechanistic effects of fluid resuscitation, demonstrate the fidelity of our animal model, and encourages the use of this model for future sepsis research applications. Subsequent experiments, and additional instrumentation will only further improve upon our animal model, and will continually improve translational research and education.

3.6 Conclusion

We demonstrated the unique ability to monitor systemic physiological parameters over the course of a septic insult, and couple these data to microvascular data in the hindlimb muscle. Coupling these measurements we tested clinical fluid resuscitation protocols using both saline and 5% human albumin, two of the most commonly used, and commonly debated fluids. Our preliminary findings indicate that albumin is better at
restoring macrovascular homeostasis, as well as restoring microvascular perfusion and oxygenation in sepsis.
3.7 References


Chapter 4

Contributions, Limitations and Future Directions

4.1 Contributions

Recently, there has been a call for animal models of sepsis that are more representative of the clinical scenario. There is a noticeable disconnect between preclinical experimental findings, and successful translation to the clinical environment, which is illustrated in the failure of many multi-center clinical trials. While success in human patient cohorts is important, animal models remain as essential tools in developing new therapies and determining the mechanism of clinical interventions.

While many models of sepsis are currently in use, each with distinct advantages, there is still a need for high-fidelity animal models that can be adapted depending on the needs of critical care[5].

In addition, the ability to provide macrovascular and microvascular data, in conjunction with a controlled septic insult, is of utmost importance, especially considering that the microcirculation is being recognized as an important target for early goal-resuscitation therapies[15, 33].

The goal of this thesis was to develop an animal model of sepsis, building on the recommendations of ICU physicians, that is more relevant to the clinical septic response. We have demonstrated a dual-sided approach that provides concurrent macrovascular and microvascular data, over the entire course of a FIP-induced sepsis insult. Our use of FIP to induce sepsis allows for a greater degree of control than other more variable models such as CLP, which is still considered the gold standard. The commonality of the CLP lies in the ability to replicate the events associated with the early phase of a septic response (low CO, high TPR), which our FIP insult is also able to reproduce. The unique ability of our model lies in our high-level instrumentation, which provides physiological data similar to what would be available for a patient in the ICU. Our use of a dual-wavelength camera allowed us to extract valuable quantitative microvascular data, which provides insight into the environment that may be surrounding the vital organs. As
current clinical microvascular data uses the sublingual microcirculation to assess degree of dysfunction, we are able to apply the same principles in an organ remote to the site of initial infection. A further advantage of our model lies in the ability to extract oxygen saturations for individual capillaries, effectively removing reliance on purely qualitative-based assessment of microvascular perfusion. This aspect of our model has many implications in terms of future applications of the model aimed at determining the mechanistic effect of therapeutics. Applying our model in this manner was the secondary goal of this thesis.

The secondary aim of this thesis was to apply our developed model in an attempt to address the efficacy of saline and albumin as resuscitation fluids. Guided by our research colleagues (Dr. Michael Sharpe, University Hospital, London, ON – Critical Care and Anesthesia, and Dr. Alison Fox-Robichaud, McMaster Hospital, Hamilton, ON – Internal Medicine, Critical Care) and the Canadian Critical Care Translational Research Group, we decided that the first application of our model would test the efficacy of fluid types using current clinical diagnostic criteria. In our preliminary study we administered either saline or albumin in order to maintain systemic blood pressure of 75mmHg or greater. We demonstrated our ability to illustrate the mechanistic effect of fluid type on macrovascular and microvascular environment in sepsis. While saline and albumin were both able to restore blood pressure, the trends at the 5-hour timepoint for other systemic parameters were different. Saline was unable to improve cardiac output, stroke volume, and maintained increased TPR, while albumin had the opposite effect, increasing cardiac output, and stroke volume, and decreasing TPR. The situation at the microvascular level was similar. In terms of oxygen saturation, velocity, and supply rate, saline animals declined over the course of the response, while the animals in the saline group increased after administration of an albumin bolus. These results suggest that albumin may provide a therapeutic benefit beyond hemodynamic optimization, and is more effectively at restoring systemic homeostasis than saline.

In addition to providing evidence for the use of albumin in fluid resuscitation, we developed a fluid resuscitation model of sepsis that can be tested in conjunction with
other therapies, co-morbidities, and disease severities to further increase the translational ability of our model.

This translational tool has several potential applications, and addressed many of the drawbacks of previous models that have been recently examined[1]. This model has several potential additions that will strengthen its integrity, and it is well set-up for modifications, as needed, to meet the demands of critical care research.

4.2 Limitations/Additions

The limitations of these studies are mainly technical, and involve the instrumentation of the rat in our protocol. Some of these were alluded to in the discussion of the previous chapters.

Since we are mechanically ventilating the rat with a preset ventilation rate (generally 73-76 breaths/min), applying a positive end expiratory pressure (PEEP) would be a valuable addition to our model. Currently we relied on the PaO₂ and PaCO₂ values measured via the iSTAT cartridges and arterial blood samples to determine the ventilation rate, but these measurements provide little indication about respiratory distress. By changing the ventilator that we use we would be able to apply a PEEP within our animals. The application of PEEP would be valuable to ensure airways stay open, which would offset acute respiratory distress syndrome (ARDS), and respiratory failure due to edema formation.

Second, catheterization of the bladder to monitor urine output is another potential addition to our model, which would provide another measurement common in clinical practice. While we did not include this procedure in our current protocol, the technique to catheterize the bladder of a male within the peritoneal cavity has recently been developed[4]. Due to our use of pentobarbital anesthetic this catheterization technique is necessary, as urine does not passively flow, as the central nervous system is depressed. This technique would be a valuable addition to the model, and would provide insight into the degree of kidney dysfunction in the rat, adding another aspect of clinical inclusion criteria. We make a midline incision for the implantation of the cardiac output flow
probe, which makes the bladder catheterization accessible. The reason that we did not include this in the original model is due to the difficulty of surgery, and the fact that the suggestion of this technique came after several animal experiments were completed. Due to the increased inflammation that would occur due to the extra surgery, we did not want to introduce unnecessary variability into our experiment.

Next, measurement of mixed venous saturation would improve upon the clinical relevancy of our model. Mixed venous oxygen saturation is another target common to early-goal directed therapies, and is often used as a metric of imbalance between oxygen delivery and systemic oxygen consumption[3]. Although we rely on MAP to guide our resuscitation protocols, it would be worthwhile to monitor consistent changes in mixed venous oxygen saturation to get a sense of how accurate reliable it is as a tissue perfusion surrogate. Continuous measurement of venous oxygen saturation is possible using an implanted fiber-optic catheter, and has been demonstrated in newborn infants[5]. Barring the lack of instrumentation in our lab, we could adapt this technique for use in our model, as we already catheterize the jugular vein, thus extra surgery would not be required.

Measurement of white blood cell count during the experiment would be another valuable addition to our model in terms of clinical indications of sepsis (WBC ct. ≥12 or ≤4). Performing a complete blood count at each timepoint could provide information about platelets as well, which is another measurement common to the bedside. Currently this test is not performed due to time constraints in the experimental protocol, however could be added with the help of additional personnel.

Another addition that could be useful in terms of our model would be if we had the ability to monitor the amount of applied heat required to maintain core body temperature of 37°C. In the clinical setting patients becoming either hypothermic (temp <36°C) or hypothermic (temp > 38°C) is an indicator of systemic inflammation. Monitoring the amount of applied heat would provide insight into the temperature control abilities of septic animals.

In addition, given that we continuously monitor systemic parameters over the course of the septic insult, it would be interesting to look at heart-rate variability. Heart-rate
variability analysis can provide insight into myocardial distress, which could provide insight into the cardiac output trends that are seen in the septic animals. This data has already been acquired, and thus analysis of the data is possible to determine the effect of sepsis on heart rate variability in our model. This also has clinical implications as heart-rate variability can easily be measured in human patients, and may have predictive effects.

Lastly, at the end of the experiment, the vital organs were harvested (kidney, spleen, liver, skeletal muscle, diaphragm, lung, heart, and brain) and stored for future use. These tissues could be used for genomic analysis in order to measure gene expression in sepsis. Our institution has the infrastructure to analyze the entire genome of the rat (London Regional Genomics Centre, Robarts Research Institute, London, ON). This analysis could provide valuable information about which genes are upregulated in sepsis, and how the administration of fluids or therapeutics affects this regulation. This also has clinical implications as patient blood samples are often taken in an attempt to detect early markers of sepsis (e.g. IL-6, TNF-α), which can be used to aid in therapeutic decisions[6].

4.3 Future Work

In terms of future work, there are several potential experiments that involve applications of our model.

As was previously alluded to in the introduction of this thesis, the developed central model permits adaptability and introduction of co-morbidities is a logical next step application. Since septic patients commonly have co-morbidities, the ability to test the septic response in a variety of cases would provide valuable insight in terms of patient screening, and illustrate if specific cohorts of patients are better suited to specific therapies. For instance, using a diabetic animal, or an animal with cardiovascular disease, would provide valuable information about a common clinical comorbidity present in septic patients.
It also follows, since the preliminary application of our model was to test albumin and saline, that we could also test other fluids, or administer blood transfusions. In this way we would be able to deduce which fluid best achieves clinical targets, as well as restoring microvascular perfusion. Along the same lines, testing concomitant therapies would be another useful application of this model and would improve clinical adaptability, as patients commonly receive more than just fluids for treatment (e.g. vasopressors, inotropic agents, antibiotics)[7].

It would also be valuable to demonstrate the validity of our use of the EDL as representative vasculature in the vital organs. It would be valuable to use near infrared spectroscopy (NIR) in easily accessible vital organs (kidney, brain) in order to assess changes in oxygen saturation over the course of the septic response.

Since platelet aggregation is associated with the inflammatory response, the ability to visualize platelets in conjunction with our dual-wavelength technique for capillary oxygen saturation measurements would provide valuable information. This would be especially useful for fluid resuscitation, as albumin is believed to have anti-inflammatory properties, which this technique would be able to visualize. This could be accomplished through the use of spinning disc confocal microscopy, and fluorescent labeling of platelets. After the dual-wavelength videos were captured, the cameras could be switched to the fluorescent channel, and the platelets in the same channel could be visualized.

Furthermore, as sepsis is associated with endothelial dysfunction, which causes fluid to leak out of the vasculature, it would be interesting to be able to monitor the degree of leak in sepsis, and compare the effect that fluid resuscitation has on edema formation. Using a dye such as Evans blue would permit this measurements, as it can be injected into the animal ~2 hours before sacrifice (which corresponds to onset of sepsis in our model and after the animal is sacrificed, the tissues could be homogenized, and the dye concentration determined using a spectrometer. This experiment would provide insight into the areas of the animal that are most susceptible to vascular leak, and allow us to see where resuscitation fluid was collecting. This experiments would further our comparison
between resuscitation fluids, as the interaction of specific fluids and the edematous tissue could be considered.

Lastly, this model could serve as a valuable education tool, as we were able to effectively create a miniature ICU. Clinicians or residents hoping to gain experience in treating a septic patient would be able to obtain real-time training, and would be able to intervene, as they deem necessary, based on the same parameters that are available at the bedside.
4.4 References


Appendix A: Animal Ethics Approval

Western

2007-061-08::6:

**AUP Number:** 2007-061-08

**AUP Title:** A Systems Approach to Sepsis: Pathological versus Adaptive Microvascular Response

**Yearly Renewal Date:** 10/01/2013

The **YEARLY RENEWAL** to Animal Use Protocol (AUP) 2007-061-08 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Appendix B: Camera Set-Up, Flow-Probe Measurement

Dual-wavelength intra-vital video microscopy

In our experiment we use dual wavelength microscopy to take advantage of the differences in absorption spectra of oxy- and deoxyhemoglobin at specified wavelengths (Fig. 4-1). The extraction of capillary SO$_2$ from the dual-wavelength capture is based on the equation for the optical density of whole blood at a particular wavelength given by:

$$\text{OD}_\lambda = \varepsilon_\lambda^1[HbO_2]d + \varepsilon_\lambda^0[Hb]d$$  \hspace{1cm} (1)

Where $\varepsilon_\lambda^1$ is the extinction coefficient of oxyhemoglobin at the selected wavelength, $\varepsilon_\lambda^0$ is the extinction coefficient of deoxyhemoglobin at the same wavelength, $[HbO_2]$ is the concentration of oxyhemoglobin, $[Hb]$ is the concentration of deoxyhemoglobin, and d is the path length of light.

By measuring the optical density in our video captures images at two wavelengths (442-nm – large absorption difference, 454-nm – no absorption difference) we can calculate the ratio of the optical densities, and hence the oxygen saturation via the equation:

$$S\text{O}_2 = m \left(\frac{\text{OD}_M}{\text{OD}_I}\right) + b$$  \hspace{1cm} (2)

where $\text{OD}_M$ is the optical density from (1) where there is a large difference in absorption (442-nm), $\text{OD}_I$ is the optical density from (1) where there is no difference in absorption (454-nm), and the slope, m, and intercept, b, can be calculated by substituting the $\text{OD}_M$ and $\text{OD}_I$ in the form of (1) and the fact that $S\text{O}_2 = \frac{[HbO_2]}{[H_T]}$ that is, the concentration of oxyhemoglobin divided by the total concentration of hemoglobin[1].
White light from a xenon light source transilluminates the capillary network, and the image is duplicated using a beam splitter, with band-pass interference filters for the selected wavelength (Fig.4-1), and subsequently captured with two separate digital cameras. The images are then post-processed to generate functional images by methods developed by Japee et. al. [2].

The functional images are used by proprietary software developed in our laboratory to trace the in-focus capillary and create space-time images (S/T images). These images are created based on the location of the capillary wall, and the location of the RBCs within the capillary column. The space-time image is a time series plot of the movement of RBCs through the capillary over the course of the video capture. The created plots illustrate the change in location of RBCs in the capillary with respect to time, and can be used to measure RBC velocity (μm/s) in capillaries based on the frame-by-frame displacement of RBCs and plasma gaps over the course of the video capture. Supply rate (# of cells/s) can also be deduced based on the velocity of the RBCs in the column and the lineal density (RMCs/mm), which is based on spatial average of optical density over the capillary length. The calculations are carried out using proprietary analysis software developed in our laboratory that utilizes previously developed techniques to extract capillary data for analysis of the microvascular environment [3]. The measurements that we consider include: velocity, oxygen saturation, supply rate, and capillary diameter. A pictorial representation of capillary data extraction is provided (Fig. 4-2).
Figure 4-1 Absorption spectra of human hemoglobin. The superimposed lines indicate the wavelengths that our camera capture system utilizes. The 454-nm wavelength is isosbestic, and the 442-nm wavelength is differentially absorbed by oxyhemoglobin (HbO₂) and deoxyhemoglobin (Hb).
Figure 4-2 Experimental camera capture, and analysis set-up. During the experiment, dual-wavelength intra vital video microscopy is used to monitor capillary blood flow in the hindlimb muscle of the rat. The dual wavelength capture (20X) is then post-processed to create SAD images, which are used by the vessel selection GUI to trace the in-focus capillaries, and create space-time (S/T) images. The S/T images are then used by the vessel analysis GUI to calculate changes in velocity, hematocrit, supply rate, and oxygen saturation over time, using the data contained in the S/T image. This process is repeated
for the in-focus capillaries in 10 fields of view to assess the global tissue microcirculatory environment.

**Transonic Flow Probe Cardiac Output Monitor**

The flow probe that was used to monitor cardiac output was a Transonic 2PSB (2mm) probe. The probe head consists of two ultrasonic transducers attached on a cuff that are positioned on one side of the vessel being monitored. The cuff on the probe head is associated with a metal reflector that is fixed in place on the side of the vessel opposite the transducers. The reflector acts to redirects the ultrasound signal emitted from one of the upstream transducers, through the vessel to the upstream transducer. The time it takes the signal to traverse this path is associated with the flow of the vessel within the cuff (Fig. 4-3)
Figure 4-3 Schematic representation of transonic flow probe used to monitor cardiac output. The transducer heads emit a wide ultrasound beam, passing signals back and forth to monitor the transit time in the upstream and downstream directions. The difference in these measurements reflects the volume flow of the vessel.
Both upstream, and downstream transit time measurements are made, which is carried out by reversing the transmit-receive sequence between the transducer heads. The output cardiac output signal is the difference of the upstream and downstream transit time, as the upstream signal is going against the “current”, while the downstream signal is going with the “current” thus each metric is needed for signal accuracy. Each transducer emits a wide-beam signal, which ensures that the volume flow calculation is accurate. The fact that the beam intersects the vessel twice (once on transmission, and once on receiving end) decreases the effect of diameter differences and vessel alignment issues, thus allowing for reliable implantation and measurements.

There are a few limitations to the use of the cardiac output probe. These include the placement of the probe anatomically, and the use of anesthetic. Since cardiac output is a measure of the total volume of blood pumped from the heart per minute, the further away from the aorta the greater the misrepresentation of the measurement. Thus our use of measurement on the abdominal aorta does not include flow to the brain, or flow in the brachial arteries and forelimbs. Next, it has been shown that pentobarbital anesthetic depresses cardiac output in rats, and thus it will cause cardiac output to be lower than expected. For these reasons it makes sense to measure relative change in cardiac output from baseline, rather than attempting to compare cardiac outputs across groups due to the high potential for confounding variables.
References


Appendix C: Protocol Modifications

The first chapter describing the development of the animal model underwent several protocol modifications. This appendix will describe these modifications, the associated problems with each objective, and the most effective resolutions. This appendix aims to mitigate these obstacles for trainees hoping to utilize this animal model in the future.

Modification 1 – Non Fluid-Resuscitated CLP

The first objective in the development of this model was to fully instrument the animal in order to consistently obtain macrovascular measurements (MAP, HR, CO, CVP, Urine Output). Next, we initiated sepsis using the CLP method. The EDL was then blunt-dissected as described in Chapter 2, and the animal was transferred to the microscope stage. The total number of rats used in the development of this preliminary model was eighteen (n=18). The number of animals attributed exclusively to each challenge is indicated.

Challenges

Probe (nprobe = 6): The placement of the flowprobe was the source of several issues as manipulation of the inferior vena cava is a fragile process. The process of separating the connective tissue that attaches the abdominal aorta and the inferior vena cava is difficult and requires careful surgery. Furthermore, upon separation of the membrane the direction that the flowprobe cuff encircles the abdominal aorta is important. Both of these issues were considerable sources of difficulty in the preliminary stages of this model and were addressed as follows:

1) To separate the membrane between the inferior vena cava and the abdominal aorta, the first step is to use cotton swabs to separate the superficial membranous tissue. Once this is complete, blunt, curved forceps must be used to slightly pick up the abdominal aorta, from the right (anatomical left) side. The forceps tips should be seen through the connective tissue (between the vena cava and abdominal aorta), when viewed through a surgical microscope. Continuing to use the surgical microscope, a pair of serrated straight forceps must be used from the
left (anatomical right) side to tease away the connective tissue, in between the vena cava and abdominal aorta, with the majority of the force applied to the tips of the blunt curved forceps lying underneath. The force must be applied to the tips of the forceps favoring the left side, towards the vena cava, as force in the opposite direction (towards the abdominal aorta) will cause the vena cava to tear, effectively ending the experiment.

2) Once a hole is made in the membrane, the process should be continued vertically until the hole is big enough to fit the flowprobe cuff. After the hole is made, the flowprobe cuff must encompass the abdominal aorta by first being inserted through the hole, underneath the aorta, and exiting out the right (anatomical left) side. This specific direction is needed such that when the animal is rotated on the stage the aorta will fall into the probe head, and will not slip out when the animal is transferred to the stage.

CLP ($n_{\text{CLP}} = 4$): The CLP surgery was the source of problems, as the procedure requires manipulation of the cecum, tying off of the supplying blood vessels, perforation of the membrane, and palpation of the cecum to release fecal contents. Besides the surgical challenge, it is difficult to exactly replicate the amount of feces that is extruded. Furthermore, the surgery is not possible to perform on the microscope stage, and thus the time between initiation of insult, and transfer to the stage is sufficient such that true baseline measurements cannot be obtained. Due to this transfer time, the initial measurements were made at least one hour after the initial insult, and thus do not represent a true baseline. This issue was addressed via the use of FIP instead of CLP to induce sepsis. Using a FIP model allowed us to transfer the animal to the stage, and then administer the initial insult, significantly reducing the time between insult and baseline. Adapting the insult in this way provided us with baseline measurements that were as close as possible to a baseline.

Urine Output ($n_{\text{Urine}} = 4$): As urine output is used clinically as an indicator of renal failure, we attempted to catheterize the bladder of the rat in order to add this measurement to our model. This surgery is invasive and it was difficult to find a detailed
protocol describing this method. Due to several failed surgical attempts, and the associated termination of data acquisition, the catheterization of the bladder was abandoned as an addition to the model. Further training may be required in order to add this measurement to the model.

**Video Recordings (nvideon=18*)**: The video recordings are an integral part of our model because they allow us to monitor changes in the microcirculatory environment in the EDL over the entire course of the septic insult. While the protocol modifications eventually led to baseline video captures immediately following septic insult, we eventually realized that we needed to add a middle timepoint in order more effectively track the progression of sepsis at the microcirculatory level. *Note: due to the fact that the video capture protocol does not affect animal survivability, this n-value indicates the number of animal experiments that were carried out without a middle (3-hour) timepoint.

**Conclusion**

In developing this preliminary model we realized the importance of a true baseline when considering the septic response. We also realized the importance of consistent placement of the abdominal flowprobe to prevent premature termination of the experiment due to the surgical procedure. We also realized the difficulty required to obtain urine output measurements, and thus sacrificed this addition to the model. Lastly, we realized the importance of the 3-hour timepoint in order to better track the microcirculatory response to the septic insult.

**Modification 2 – Resuscitated FIP Model**

The second objective after the initial development of the model was to begin implementing clinically relevant resuscitation strategies upon diagnosis of sepsis. This secondary protocol underwent several modifications regarding the severity of the initial insult (FIP), the criteria for diagnosing our animal as septic, and the procedure for providing fluids to the animal. The total number of rats used in the development of this model was eighteen (n=18). The number of animals attributed exclusively to each challenge is indicated.
Challenges

**Sepsis diagnosis (n\textsubscript{Diagnosis}=4):** The diagnosis of our animals as septic was an important addition to this model, and was based on input from ICU physicians. In the first stages of this model, the animal was considered septic at the 3-hour timepoint (not based on systemic parameters). This protocol was adapted such that sepsis diagnosis was based on mean arterial pressure dropping below 75 mmHg consistently for 5 minutes. This adaptation was very important to increase clinical relevance, as we introduced sepsis diagnosis based on common clinical inclusion criteria. The clinical inclusion criteria is MAP \leq 65 mmHg. The reason for the increase used in our model is explained in the next section regarding the severity of the FIP insult. Briefly, animals in the untreated FIP group would not survive to the 5-hour timepoint if the insult was severe enough to drop blood pressure below 65 mmHg around the 3-hour timepoint of the experiment. This adaptation was also very important in terms of guiding our fluid-resuscitation strategy. We would begin administering fluids to animals in the treatment groups upon diagnosis of sepsis, and would continue to provide fluids until the animal’s blood pressure could be maintained (MAP \geq 75 mmHg).

**Severity of FIP (n\textsubscript{Severity}=10):** The severity of the initial insult had to undergo continual modification as the FIP animals had to become septic, and also survive until the 5-hour timepoint. This process was especially difficult in the non-resuscitated FIP group, as the untreated animals would rarely survive until 5 hours if sepsis was diagnosed around the 3-hour timepoint. This step in the protocol involved several modifications to the volume of the FIP insult; too little insult would result in a non-septic animal, whereas too large of an insult would cause the premature termination of the experiment. Eventually an effective dose for the sake of our experimental protocol was realized, which is presented in Chapter 2.

**Gradual vs. Bolus Fluid-Resuscitation (n\textsubscript{Gradual}=4):** For the animals in the resuscitation group, we initially began resuscitation via a gradual infusion of fluid, rather than a bolus injection. Upon discussion with our collaborators (ICU physicians) we decided that bolus injections were more clinically relevant. Thus the final modification to the protocol was
to resuscitate the animals to achieve the clinical target (MAP ≥ 75 mmHg) using successive 1 ml bolus injections as needed.

**Conclusion**

In the development of this secondary model we realized the importance of diagnosing sepsis based on clinical criteria. We also determined that MAP ≤ 75 mmHg was effective to use as our sepsis inclusion criteria. This method also helped to ensure that our fluid-resuscitation strategy was based on a physiological parameter, and the animals were treated consistently between groups. These modifications also led to the most effective FIP insult volume such that the animals became septic during the experiment, but the untreated animals could survive until the 5-hour timepoint. Lastly, the final modification enhanced the clinical similarity of our resuscitation protocol, and we determined that bolus injections should be used to fluid resuscitate.

**Final Model – Translational FIP Model of Sepsis**

Once all of the obstacles mentioned above were overcome, we decided our protocol was clinically relevant and began applying our model to test the efficacy of fluid type on the macrovascular and microvascular environment in a rat FIP model of sepsis. The number of animals included in the final model was twelve (n=12) and are presented in this thesis.
Appendix D: Curriculum Vitae

Nathaniel Edward Hayward

Education

Master of Science, Medical Biophysics
The University of Western Ontario, London, ON, Canada
(2014) (Expected)
Thesis: Development of a Translational Animal Model of Sepsis
Supervisors: Dr. Christopher Ellis, Dr. Dwayne N. Jackson

CIHR Strategic Training Fellowship in Vascular Research
Canadian Institute of Health Research, training completed at
The University of Western Ontario, London, ON, Canada
(2012)

Bachelor of Science
The University of Western Ontario, London, ON, Canada
(2011)
Major: Honors Specialization Medical Biophysics
(Physical Science Concentration)

Conference Proceedings

*Hayward NE, Sharpe, MD, Jackson, DN, Ellis CG., “Development of a translational animal model of sepsis” (Accepted), Canadian Critical Care Translational Biology Group Meeting, Quebec City, June, 2013

*Hayward NE, Sharpe, MD, Jackson, DN, Ellis CG., “Resuscitation fluid efficacy on microvascular dysfunction in a translational animal model of sepsis” (Accepted), NAVBO, Cape Code, MA, October, 2013

*Oral Presentation

Related Work Experience

Teaching Assistant
The University of Western Ontario, London, ON, Canada
(2011-2013)
Conducting undergraduate research labs, marking lab reports and assignments

Certifications

Animal Use Training Certificate
Granted by Animal Care and Veterinary Services,
University of Western Ontario, London, ON, Canada
(2011)
Basic Animal Care and Use: Rat
Anesthesia and Surgery: Rat