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Coagulation and sepsis in murine models

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COAGULATION AND SEPSIS IN MURINE MODELS

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KATHERINE INSUN WEE

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Approved by

First Reader ____

Daniel Remick, M.D.
Professor of Pathology and Laboratory Medicine

Second Reader ____

Shinichiro Kurosawa, M.D., Ph.D.
Associate Professor of Pathology and Laboratory Medicine

DEDICATION

I would like to dedicate this thesis to my advisors, family (Christian, Esther, and Shirline Wee), and friends, who have given me unconditional love and support throughout this journey of obtaining my MAMS/MPH degree at Boston University.

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COAGULATION AND SEPSIS IN MURINE MODELS

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ABSTRACT

Sepsis is a widespread and expensive health issue that annually affects millions of individuals in the United States. Despite significant research investments, there has yet to be a single proven effective therapy, resulting in the sustained high mortality and morbidity counts. Sepsis is a multifaceted and complex problem, affecting many systems in the body. Thus, steady investigations have been made to determine the mechanisms of the pathophysiology and multi-level systems involved in septic immune response.

The purpose of this study aims to determine if sepsis affects coagulation. The secondary aim seeks to reproduce findings on the effects of hydrocortisone, ascorbic acid, and thiamine triple therapy on septic mortality. This was achieved through the induction of peritonitis through cecal ligation and puncture (CLP) in murine models. Prothrombin time (PT), as well as plasma glypican (GPC) levels, were measured and analyzed in mice stratified by different septic phenotype categories. Furthermore, the non-invasive physiologic parameters were evaluated to see if there were substantive evidence of changes or other body systems being affected by sepsis and treatment method.

Results showed that there were significant differences in weight, temperature, heart rate, respiratory rate, and oxygen saturation post-CLP, in which mice predicted to die (P-Die) had lower values than mice predicted to live (P-Live). In most cases, the significant differences within P-Live and P-Die mice occurred within 6hrs post-CLP. This suggests that effective therapy includes early intervention. The PT levels revealed

there was a significant difference between VEH/P-Live ($M=15.58$, $SD=5.8$) and VEH/P-Die ($M=24.03$, $SD=133$), $p=0.0428$. This demonstrated that P-Die mice had elevated prothrombin times as anticipated.

The findings also found that GPC-1 levels were significantly elevated in mice predicted to die in both HAT-treated and vehicle groups; HAT/P-Die ($M=3658$, $SD=3516$), VEH/P-Die ($M=8845$, $SD=1966$), $p=0.0143$. This hinted at the possibility of HAT improving glycocalyx shedding. It also found that GPC-1 levels were significantly higher in VEH/P-Die ($M=8845$, $SD=1966$) when compared to VEH/P-Live ($M=1516$, $SD=1485$), $p<0.0001$. Results for GPC-4 were found to be correlated to GPC-1 levels, $r(40)=.74$. Thus, it was observed GPC-4 levels were also significantly elevated in mice predicted to die in both Hat-treated and vehicle groups; HAT/P-Die ($M=2182$, $SD=1267$) and VEH/P-Die ($M=4515$, $SD=667.3$), $p=0.0038$. And finally, GPC-4 levels were elevated in VEH/P-Die mice ($M=4515$, $SD=667.3$) compared to in VEH/P-Live mice ($M=2033$, $SD=480.3$), $p<0.0001$.

Therefore, the study found that P-Die Mice have increased PT and elevated GPC levels. This can be seen in association between the activation of coagulation leading to consumptions of coagulation factors, including II, V, VII, and X, and the breakdown of the endothelial barrier, respectively. Further research is necessary to investigate the robust coagulopathy system of sepsis, organ dysfunction, and HAT, or hydrocortisone, as a potential treatment for those with poor predicted outcomes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Bpm	Beats Per Minute
CLP	Cecal Ligation and Puncture
DIC	Disseminated Intravascular Coagulation
ELISA	Enzyme-Linked Immunosorbent Assay
GPC	Glypicans
HAT	Hydrocortisone, Ascorbic Acid, and Thiamine Triple Therapy
HBSS	Hanks Balanced Salt Solution
Hr	Hour
HR	Heart Rate
IACUC	Institutional Animal Care and Use Committee
O2 sat	Oxygen Saturation
PD	Pulse Distention
P-Die	Predicted to Die
P-Live	Predicted to Live
PT	Prothrombin Time
RCF	Relative Centrifugal Force
RR	Respiratory Rate
TF	Tissue Factor
TFPI	Tissue Factor pathway inhibitor
VEH	Vehicle Treatment

INTRODUCTION

Sepsis – Mortality and Morbidity

The highly undue burden of sepsis warrants public health awareness and action. Every year, at least 1.7 million adults in the U.S. acquire sepsis, leading to more than 250,000 deaths (Rhee et al., 2019). In 2017, sepsis was the most expensive in-hospital condition, costing more than $38 billion (8.8% of the aggregate U.S. hospital costs), and the second most common reason for hospitalization (5.8% of all U.S. hospital stays) (Liang et al., 2020). Though it is well-known that sepsis disproportionately affects "patients who are elderly, have severe comorbidities, and have impaired functional status", the extent to how many of these sepsis-associated deaths in adults can be prevented requires additional investigation (Martin et al., 2006; Novosad et al., 2016; Prescott et al., 2014; Rhee et al., 2019). Furthermore, the lack of standard treatment combined with the difficulty for early diagnosis and intervention contribute to the continued high mortality and morbidity counts resulting from sepsis (Fisher et al., 2019).

Sepsis is currently defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016). It is a complex response that involves the timely initiation of pro- and anti-inflammatory responses, which can be accompanied by significant changes to nonimmunological pathways, including but not limited to cardiovascular, neuronal, autonomic, hormonal, bioenergetic, metabolic, and coagulation systems (Angus & Van Der Poll, 2013; Deutschman & Tracey, 2014; Hotchkiss et al., 2013; Singer et al., 2004; Singer et al., 2016). The accumulation and presentation of these changes are crucial to identify for a sepsis diagnosis. However, despite the significant research investments, there has yet to be a single therapy consistently proven to be effective.

Murine Models

Murine models make it feasible to test potential new therapies, allowing for levels of sophistication beyond the limitations often faced with cell and tissue culture (Lewis, Seymour, et al., 2016). Thus, murine models of sepsis and their relation to human disease must be taken into consideration for the future development of innovative biologic therapeutics (Lewis, Seymour, et al., 2016). Debates are centered around the substantial genomic differences between rodents and humans, questioning the appropriateness for translational research that is intended for human-centered patient therapy (Efron et al., 2015; Seok et al., 2013). In rodents, sepsis has been cured in different ways, but none have been translatable to humans thus far. This is why model selection is crucial to draw appropriate comparisons.

Murine models are often selected among the varying animal models of sepsis because they are easy to use for experiments, there is an extensive availability of genetically engineered murine species, and they are relatively inexpensive models (Lewis, Seymour, et al., 2016). Although the perfect murine model of sepsis does not exist, the CLP-induced murine model presents as a good model for the focus of this research (Lewis, Seymour, et al., 2016). The standardized murine model protocols for cecal ligation and puncture (CLP)-induced sepsis in literature allow for comparison and attempt to accurately imitate the conditions of human clinical trials. This is accomplished with the inclusion of antibiotics, which are representative of the standards of patient care, and the stratification of the experimental host responses to septic insult. This innovative categorization ensures that testing therapies are not accomplished in “mice that have gone past the point of salvage or in animals that have not developed a clinically substantial response to the infection” (Deitch, 1998; Deitch, 2005; Lewis, Seymour, et al., 2016; Lewis, Yuan, et al., 2016; Osuchowski et al., 2009).

Cecal Ligation and Puncture (CLP)

CLP is validated and widely used among the many murine models of sepsis which mimic the pathophysiology of human sepsis (Howell et al., 2013; Hubbard et al., 2005; Lewis, Yuan, et al., 2016; Wichterman et al., 1980; Zhang et al., 2011). CLP involves a laparotomy under general anesthesia and the surgical perforation of the ligated cecum of mice via needle puncture (Leon et al., 1998; Remick et al., 1998). This procedure allows for the immediate and continuous drainage of cecal bacteria into the peritoneal cavity. The three resulting effects to the host from CLP include: 1) surgical trauma to the tissues, 2) ischemic tissue resulting from ligated cecum and devascularization, and 3) polymicrobial infectious sepsis from fecal spillage after cecal puncture (Dejager et al., 2011; Ebong et al., 1999; Lewis, Seymour, et al., 2016; Lewis, Yuan, et al., 2016). Although a relatively simple procedure, severity of polymicrobial septic peritonitis has significant variability due to factors including but not limited to puncture size and quantity of the needle, dietary status of the mouse, anesthetic protocol differences, amount of stool extruded from the cecostomy, time of day that the procedure occurred, and the variability in incision size and surgical technique (Hubbard et al., 2005; Lewis, Seymour, et al., 2016; Lewis, Yuan, et al., 2016; Newcomb et al., 1998; Rittirsch et al., 2007). This versatility in adapting to and experimenting a range of severity and testing objectives makes it favorable but may also detract from standardization of procedures between laboratories.

The physiologic response and changes to CLP-induced sepsis depends on fluid resuscitation, or else the mice “hyperdynamic circulatory state” will not be activated, which increases their mortality rates (Newcomb et al., 1998; Remick et al., 1998; Seely et al., 2011; Vianna et al., 2004). It is well-documented that mice experience hypothermia after CLP-induced sepsis (Lewis, Yuan, et al., 2016; Remick et al., 1995; Tao et al., 2004). Therefore, it is necessary to include routine and specific antibiotic administration because it can substantially alter the model (Lewis, Seymour, et al., 2016). Studies have shown antibiotics can impact the septic mice’s survival, posing great risk reduction as the degree of severity increases (Turnbull et al., 2004). Another variable directly related to overall survival is the timing of antibiotic administration. One study showed that when antibiotics were administered 12hrs post-CLP, mice with IL-6 concentrations greater than 14,000 pg/mL had 0% survival; administration of antibiotics at 6hrs to mice with similar IL-6 concentrations had an increased overall survival of 25% (Lewis, Seymour, et al., 2016; Vyas et al., 2005). This finding proved that consistency and timeliness of antibiotic administration is vital in order to allow for experimental comparison (Lewis, Seymour, et al., 2016).

It is also to be noted that the small circulating blood volume poses to be a challenge in murine models, but this issue can be overcome through the usage of micro-methods or by simply increasing the sample size. This results in a heterogeneous sample population with varying stages of the host response conducive to the experimental design (Lewis, Yuan, et al., 2016; Poli-de-Figueiredo et al., 2008).

HAT Therapy

With investments in sepsis research for potential therapy, the introduction of hydrocortisone, ascorbic acid, and thiamine triple (HAT) therapy in 2017 was pivotal. A small clinical study reported HAT significantly reduced sepsis mortality in patients. The finding met with much enthusiasm triggered more than a dozen clinical trials because if true, this could prove to be a cost-effective treatment (Kim et al., 2020). However, there was a lack of publications and studies investigating the potential mechanisms involved in HAT treatment in a preclinical sepsis model (Kim et al., 2020).

For this thesis, the efficacy of HAT therapy was tested, utilizing the prominently established CLP-induced murine model of sepsis. Previous publications suggested that noninvasive physiologic measurements may be utilized to observe the response to different therapies and temporal changes in this model (Bauzá & Remick, 2015; Mella et al., 2017). This study designed an novel method of stratifying the mice based on cardiovascular parameters (heart rate) from the 6hr data collection into predicted to live (P-Live) and predicted to die (P-Die) groups. These groups were further randomized to receive HAT treatment or vehicle (VEH). It was hypothesized that examining the effects of HAT therapy on mortality could aid in determining parameters and potential mechanisms along various affected physiological and immunological pathways. The stratification of sepsis patients for a precision medicine approach allows for the further validation of this experiment's clinical significance (Kim et al., 2020; Rello et al., 2018).

Disseminated Intravascular Coagulation (DIC)

During sepsis, there are significant alterations to the complex clotting cascade (Esmon, 2006). These changes are multifaceted, involving multiple levels within the coagulation system and the cells regulating it: clotting is initiated, the activity of natural anticoagulant mechanisms decreased, and fibrinolytic system impaired (Esmon, 2005; Remick, 2007). The coagulation cascade is an innate immunological response to bleeding caused by tissue injury (see Figure 1).

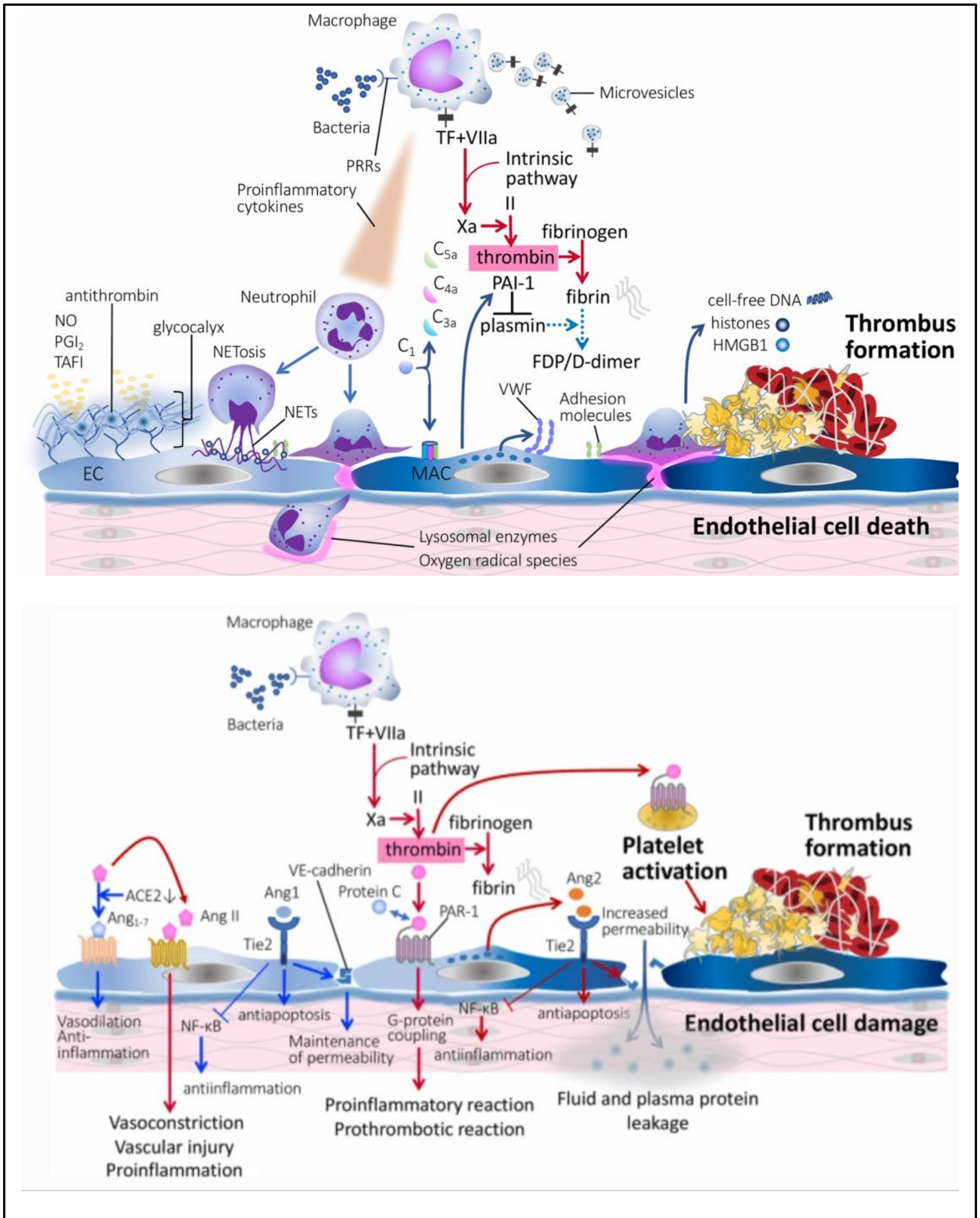

Figure 1. Pathogenesis of sepsis-associated coagulopathy. Adapted from *Recent Advances in the Research and Management of Sepsis-Associated DIC* (Iba et al., 2021)

Blood clots block blood vessels and reduce blood flow, which could potentially result in severe multi-organ failure (Remick, 2007). During sepsis, the primary activation of coagulation is triggered by the tissue factor (TF) pathway and amplified by the intrinsic pathway (Aird, 2001). This extrinsic pathway is activated when there is an injury to the endothelial tissue expressing TF, or Factor III, to the blood (Chantrathammachart et al., 2012; Franco et al., 2000). Upon binding to the TF, the circulating Factor VII is activated, forming a TF/Factor VIIa complex (Zeerleder et al., 2005). This complex activates prothrombin, or Factor X, to thrombin, or Factor Xa (Zeerleder et al., 2005). Thrombin activates Factor V and Factor VIII, greatly enhancing the coagulation cascade (Roberts et al., 1998). TF pathway inhibitor (TFPI) quickly blocks thrombin generation by TF/Factor VIIa (Hack, 2000). However, the TF/Factor VIIa complex also activates Factor IX, which combined with Factor VIIIa activates Factor X to amplify thrombin generation (Zeerleder et al., 2005). Thrombin then cleaves fibrinogen, or Factor I, into fibrin and activates Factor XIII to stabilize clots (Zeerleder et al., 2005).

Septic patients frequently experience disseminated intravascular coagulation (DIC) (Remick, 2007). Sepsis-associated DIC is recognized as “the systemic activation of coagulation with suppressed fibrinolysis that leads to organ dysfunction and systemic intravascular inflammation” (Asakura, 2014; Conway, 2019; Iba et al., 2021). Essentially, all three of the classic alterations determined by Virchow’s classic triad (which are changes in “coagulability, endothelial injury, and abnormal blood flow”) are present in septic patients. (Remick, 2007).

Prothrombin (PT) Time

Diagnosis of human DIC is based on prothrombin (PT) time, platelet count, fibrinogen, and fibrin-related markers, such as D-dimer (see Table 1). This experiment

Table 1. Proposed murine DIC score. A score ≥4 indicates the presence of DIC.

Parameter	**0**	**1**	**2**
Platelets 10^9/L	>660	<660	<200
D-Dimer ng/mL	<1100	>1100	>1400
PT seconds	<21	>21	>24

focuses on one component of the DIC score – PT time. The inclusion of PT time in this scoring system is due to the suppression of fibrinolysis seen in septic patients plays a major role in accelerating the prolonged prothrombin times (Iba et al., 2021). This measurement of coagulation disturbances is a complex and time-sensitive procedure and utilizes the plasma. However, there are limitations in using the PT time. The plasma-based testing of coagulation disregards the platelet contribution to thrombosis (Hoffman & Monroe, 2001; Simmons & Pittet, 2015). Thus, all components (PT, platelet count, and D-dimers) must be taken into consideration.

Glypicans (GPC)

Normal endothelial cells that are intact exhibit anti-coagulant properties through anticoagulant molecules and further function as a barrier between blood products and procoagulant molecules in the extracellular matrix (Remick, 2007). The endothelium is covered by the glycocalyx. The glycocalyx is composed of the complex extracellular gel of loosely attached membrane-bound proteins and glycans. (Reitsma et al., 2007). The glycocalyx also serves in other numerous roles such as maintaining vascular homeostasis. For example, it is able to sense shear stress, provide receptor sites for signaling molecules, has an antithrombotic surface, and stabilizes the barrier by generating a negatively charged fiber matrix (Alphonsus & Rodseth, 2014; Bansch et al., 2011; Chelazzi et al., 2015; Fisher et al., 2019).

Proteoglycans, proteins with glycosaminoglycan side chains, largely comprise the foundation of the glycocalyx (Chelazzi et al., 2015). Normally, most of the endothelial proteoglycans are secreted, whereas the syndecans and glypicans stay bound to the membrane (Bansch et al., 2011). There are four syndecans which all contain the glycosaminoglycan species heparan sulfate and are located bound to the cell surface through a transmembrane domain. The six different glypicans also mainly consist of heparan sulfate but are usually attached to the membrane phospholipids through a glycosylphosphatidylinositol anchor (Fisher et al., 2019; Reitsma et al., 2007). The removal of proteoglycans from the cell membrane occurs via shedding (Iozzo & Schaefer, 2015). This removal process can be triggered by pro-inflammatory factors from the host and infecting pathogens which are commonly seen in sepsis (Park et al., 2004; Schött et al., 2016). For example, some bacteria have been observed to transmit enzymes which directly shed the components of the glycocalyx (Fisher et al., 2019).

The pathophysiology of sepsis involves “the breakdown of endothelial barrier function, leading to vascular leak, edema, and organ failure” (Fisher et al., 2019; Ince et al., 2016; Opal & van der Poll, 2015). Though it is strongly thought that the endothelial cells are dysfunctional in septic patients, strong conclusions of mechanisms are difficult as it is challenging to obtain clear-cut documentation during in vivo settings (Remick, 2007). For example, it is largely unknown whether glypicans are concurrently shed with syndecans during sepsis (Fisher et al., 2019). However, it is widely known that other cells within the body lose their normal functionality. Thus, it can be observed that septic patients present with an "increased apoptosis of dendritic cells, macrophages or monocytes, and mucosal epithelial cells" (Remick, 2007). These glypicans can be detected and measured through the enzyme-linked immunosorbent assay ELISA).

SPECIFIC AIMS

The prominence of sepsis has led to an influx of studies. However, without a full comprehension of mechanisms contributing to the pathophysiology of sepsis and its manifestations, there cannot be an identification of the most effective therapy. The primary aim of this study seeks to determine if sepsis affects coagulation. This hypothesis was assessed through prothrombin time measurements and plasma glypican levels in sepsis-induced murine models via cecal ligation and puncture. Furthermore, the experimental study design originated in previous studies' findings on the effects of hydrocortisone, ascorbic acid, and thiamine triple (HAT) therapy on septic mortality. Thus, in replicative fashion, this study evaluates the validity and reliability of the physiological parameters affected by HAT treatment in sepsis-induced murine models.

METHODS

Experimental Design

Pre-clinical sepsis models were published in the past, following recommendation #13 to obtain as much information as possible (Bauzá & Remick, 2015; Chiswick et al., 2015; Kim et al., 2020; Libert et al., 2019; Mella et al., 2019; Osuchowski et al., 2018). These set the guidelines for this experimental design. The studies were conducted over 4 months on groups of 5 or 10 mice each week. To eliminate potential selection biases, both male and female, adult outbred ICR mice were used.

Baseline

Baseline information was collected from each mice in compliance with the recommendation concerning preclinical sepsis models (Osuchowski et al., 2018). Mice were first weighed, and after shaving a quarter-sized portion of the abdominal fur, the body temperature was recorded using an FDA-approved infrared body temperature thermometer (TempIR JXB-183). Physiologic measurements were then noninvasively obtained with a cervical collar (MouseOX Plus). The attached software then produced a collection of data that included heart rate (HR), respiratory rate (RR), oxygen saturation (O2 sat), and pulse distention (PD). 5 repetitions of data collection were performed in order to ensure data reproducibility. Results were further averaged to yield parameters intended for analysis. Physiologic parameters as aforementioned were continually measured noninvasively at 6 hours (hrs) and 24 hrs post-cecal ligation and puncture (CLP).

CLP-Induced Sepsis

The standard cecal ligation and puncture (CLP) induced peritonitis was performed in mice using prior protocols with minor modifications (Bauzá & Remick, 2015; Craciun et al., 2014; Kim et al., 2020). Following recommendations by the Boston University Institutional Animal Care and Use Committee (IACUC) and protocols established by prior experiments, buprenorphine (0.05mg/kg) was administered 20 minutes prior to surgery. This dosage was found to not induce any cardiac or activity depression which could potentially disturb the necessary physiologic measurements for analysis (Kim et al., 2020). After isoflurane anesthesia, CLP was performed. Mice were randomized, in which half the mice had the cecum punctured with a 16-gauge needle and the other half with an 18-guage needle. This assortment allowed for differing severities of sepsis. Immediately following surgery, mice were resuscitated with 1.0mL of warmed normal saline. At 2hrs post-CLP, antibiotic therapy was provided with imipenem (25mg/kg) in Lactated Ringers with 5% dextrose. Subsequently, 1.0mL of the imipenem therapy was administered subcutaneously per mice. This methodology was continued in conjunction with HAT treatment at 6hrs and 12hrs post-CLP. Note the imipenem and Lactated Ringer mixture is the vehicle (VEH) treatment, a positive control, that allows for comparison with hydrocortisone, ascorbic acid, and thiamine triple (HAT) treatment for severe sepsis and septic shock.

6hrs Post-CLP: Mortality Prediction and HAT Treatment

Early measurements in sepsis allow for more effective therapeutic interventions (Osuchowski et al., 2006). Thus, physiologic parameters (HR, PD, RR, O2 sat, weight, and temperature) were taken 6hrs post-CLP and were used to predict 5-day mortality. Mice were stratified into 2 groups based on the 6hr HR. Mice with HR greater than 620 beats per minute (bpm) were predicted to live (P-Live) and mice presenting with HR less than 620bpm were predicted to die (P-Die). These limits were in correspondence to previous mortality studies in CLP-induced murine models. After stratification, mice were randomized to receive hydrocortisone, ascorbic acid, and thiamine triple (HAT) therapy or a mixture of antibiotics imipenem and Lactated Ringer (VEH) treatment. This protocol created a prospective and randomized controlled experiment that increased reproducibility and rigor (Kim et al., 2020).

The original treatment protocol for patients in the clinical setting determined the HAT dosages utilized in the mice (Kim et al., 2020; Marik et al., 2017). Drugs used in treatment were prepared and measured a couple days prior to CLP. 50mL of warmed Lactated Ringer was added to 53.57mg hydrocortisone, 4.54mg ascorbic acid/vitamin C, and 1.79mg thiamine. The dissolved drugs were aliquoted, so that each 1.0mL injection of triple therapy contained 1.5mg/kg hydrocortisone, 45mg/kg vitamin C, and 100mg/kg thiamine. Mice were injected subcutaneously, and the controls continued to receive the VEH treatment. This process was repeated at 12hrs post-CLP as determined by the mortality predictions, P-Live and P-Die.

24hrs Post-CLP: Sample Collection

After the physiologic parameters were measured, mice were sacrificed with an overdose of isoflurane. Blood was collected via cardiac puncture for hematologic analysis. EDTA tubes containing 50uL of 169mM K3EDTA were used for blood collection. The peritoneum was opened and washed with 10mL of Hanks Balanced Salt Solution (HBSS) with 10 U/mL Heparin following previously established protocol (Kim et al., 2020; Mella et al., 2017).

For blood samples, whole blood was collected with 50uL of sodium citrate. 20uL of whole blood was diluted with the dilution factor 1:10 using the appropriate diluent for the automated hematology analyzer (Abaxis). After obtaining an analysis of the complete blood count, the blood was spun at a force of $2000 \times g$, or relative centrifugal force (RCF), for 10 minutes. The pellet was used for another experiment for separate analysis, and the plasma was used to document prothrombin time (PT) and further utilized in enzyme-linked immunosorbent assays (ELISA) to detect plasma glypicans (GPC).

Prothrombin Time (PT) Analysis

Maintaining the integrity of the sample and procedure is crucial because prothrombin time (PT) analysis is time- and temperature-sensitive. This is because several of the coagulation factors, Factors V, VIII, XIII, and fibrinogen, are easily broken down at $37^{o}C$ (Sigma Amelung, 2001). PT was analyzed using a semi-automated mechanical coagulation analyzer (Sigma Amelung KC4 Delta). Prior to operation, the analyzer must reach $37^{o}C$ and the reagents (Neoplastine1 and Neoplastine2) need to be solubilized into a solution. 50uL of each sample were loaded into tetravettes or micro-cuvettes, which contain stainless steel balls. Evenly coating the bottom of the tetravettes, the samples were thereafter placed into a loading rack of the instrument. After 180 seconds, the reagent was carefully dispensed, and the PT was captured. Initially for each sample, 2 rounds were performed and averaged, but due to the lack of adequate sample volume, 1 round was completed.

ELISA: Glypicans 1, and 4

In vitro measurements of glypican (GPC)-1 and GPC-4 levels were measured in the plasma of mice with CLP-induced sepsis and naïve mice, using the ELISA (Cloud-Clone Corp), specifically, double-antibody sandwich immunoassays. This procedure was run at the end of sample collection. Therefore, the frozen, aliquoted plasma had to be thawed and brought to room temperature. ELISA steps were performed per the manufacturer's directions for the kits as follows:

1. Add 100uL of standard or sample to each well. Incubate for 1hr at 37^{o}C;
2. Aspirate and add 100uL of prepared Detection Reagent A. Incubate for 1hr at 37^{o}C;
3. Aspirated and washed 3 times;
4. Add 100uL of prepared Detection Reagent B. Incubate for 30min at 37^{o}C;
5. Aspirate and wash 5 times;
6. Add 90 uL of Substrate Solution. Incubate 10-20min at 37^{o}C;
7. Add 50uL of Stop Solution. Read at 450nm immediately (Cloud-Clone Corp.).

The dilution factor applied was 1:10 for both GPC-1 and GPC-4. For GPC-1, the sensitivity was low. For this reason, calculations from a linear regression and based on the sigmoidal curve generated from the ELISA were compared to generate concentration values. GPC-4 presented with good sensitivity and specificity, and so, the calculations generated from the ELISA were directly used.

Statistical Analysis

All statistical analyses were performed with Prism Version 9.3.1. (GraphPad). Differences between P-Live and P-Die mice were analyzed using unpaired t tests and changes in parameters between time points (Baseline, 6hrs, 24hrs) for individual mice were analyzed using paired t tests. To determine differences between four defined groups categorized by treatment and mortality predictions (HAT/P-Live, HAT/P-Die, VEH/P-Live, and VEH/P-Die), one-way ANOVAs with multiple comparisons were used. Additionally, a Pearson's correlation test was performed to determine if any correlations or relationships. Finally, a multivariate linear regression was used to determine the relationship between plasma glypican levels, generating a best fit linear equation.

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RESULTS

Physiologic Changes for Septic Mice

A total of 81 mice were induced with CLP for this experiment and then further stratified into P-Live and P-Die categories based on the HR captured at 6hrs post-CLP. The descriptive statistics of the noninvasive physiological parameters at different time points are displayed in Table 2.

Table 2. Mean (SD) of Physiological Parameters

	Baseline		**6 hours post-CLP**		**24 hours post-CLP**	
	P-Live	**P-Die**	**P-Live**	**P-Die**	**P-Live**	**P-Die**
Wt(g)	29.1 (3.8)	28.2 (3.5)	31.3 (3.9)	28.4 (3.4)	29.5 (3.4)	27.3 (3.0)
Temp(ºC)	36.4 (1.1)	36.5 (0.6)	36.1 (0.4)	32.1 (3.7)	35.0 (2.7)	31.1 (5.3)
HR(bpm)	779.4 (77.1)	778.5 (47.4)	713.8 (71.4)	572.1 (82.3)	691.9 (124.7)	566.9 (179.5)
RR(brpm)	197.0 (28.4)	205.1 (27.7)	181.8 (26.5)	146.9 (29.7)	194.8 (45.7)	171.0 (51.0)
O2 sat(%)	97.1 (2.3)	97.6 (1.2)	96.7 (3.8)	98.7 (0.7)	96.0 (2.5)	97.9 (1.9)
PD(um)	502.8 (50.0)	487.8 (45.2)	294.4 (110.7)	253.9 (83.0)	331.0 (126.2)	276.9 (137.9)

Within the P-Live and P-Die groups, the weight, temperature, HR, and O2 sat decreased after CLP, while RR and PD were observed to increase between the times 6 and 24hrs post-CLP. However, these increases in RR and PD do not amount to threshold baseline measurements. As hypothesized from previous studies and physiological pathways, a general trend exists in which P-Die mice have lower value parameters as compared to P-Live mice. A closer investigation into each parameter was taken to analyze differences between groups.

Weight

Figure 2 shows the recorded weight and p-values associated between P-Live and P-Die groups at each time period – Baseline, 6hrs, and 24hrs. Unpaired t tests were performed to compare the mean weight in P-Live and P-Die mice.

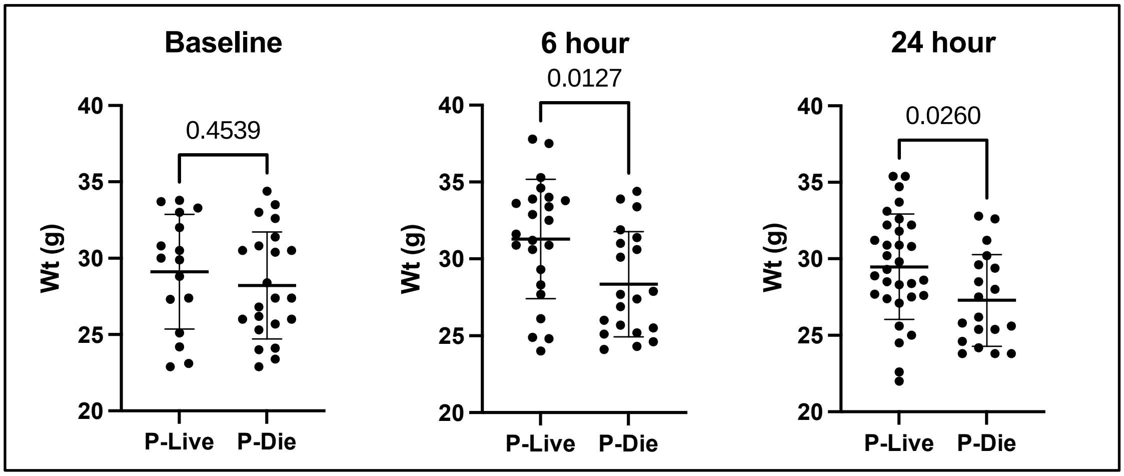

Figure 2. Weight

The analysis revealed that there was a significant difference in weight 6hrs post-CLP between P-Live (M=31.3, SD=3.9) and P-Die (M=28.4, SD=3.4); t(41)=2.606, p=0.0127. There was also a significant difference in weight 24hrs post-CLP between P-Live (M=29.5, SD=3.4) and P-Die (M=27.3, SD=3.0); t(48)=2.297, p=0.026. The unpaired t tests show P-Live mice had significantly higher weights than P-Die mice after being induced with CLP. There was no significant difference in weight between the Baseline as expected, proving the reliability of the recorded measurements.

Additionally, one-way ANOVAs with multiple comparisons were performed to compare the effect of 3 different times (Baseline, 6hrs, and 24hrs) on weight. A one-way ANOVA for P-Live mice revealed that there was no statistically significant difference in mean weight between at least 2 time points (F(2, 67)=[2.199], p =0.1183). A one-way ANOVA for P-Die mice also revealed that there was no statistically significant difference in mean weight between at least 2 time points (F(2, 58)=[0.5959], p=0.5544). Weight was not significantly affected throughout time, regardless of the P-Live or P-Die categorization.

Temperature

The recorded temperature and p-values associated between P-Live and P-Die groups at each time period are displayed in Figure 3. Unpaired t tests were performed to compare temperature in P-Live and P-Die mice.

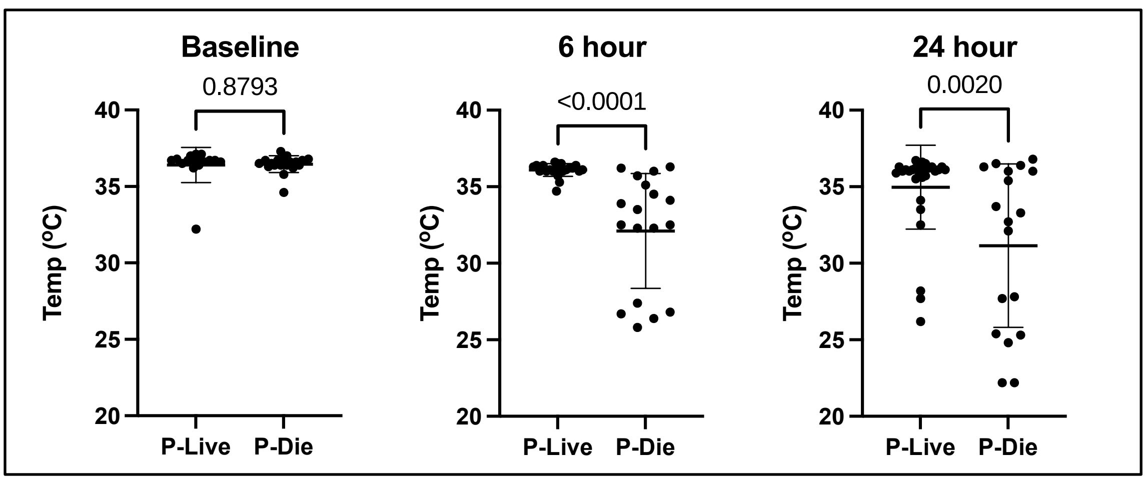

Figure 3. Temperature

There was a significant difference in temperature 6hrs post-CLP between P-Live (M=36.1, SD=0.4) and P-Die (M=32.1, SD=3.7); t(38)=4.959, p<0.0001. There was also a significant difference in temperature 24hrs post-CLP between P-Live (M=35.0, SD=2.7) and P-Die (M=31.1, SD=5.3); t(46)=3.276, p=0.0016. There was no significant difference in weight between the Baseline as anticipated, and we observe the trend that sepsis significantly leads to temperature reduction in P-Die mice at specific time points.

One-way ANOVAs with multiple comparisons were performed to compare the effect of 3 different time points (Baseline, 6hrs, and 24hrs) on temperature. The results revealed that there was a statistically significant difference in mean temperature between at least 2 time points within P-Live mice ($F(2, 65)=[3.728]$, $p=0.0293$). Tukey's HSD Test for multiple comparisons found that the mean value of temperature was significantly different between Baseline and 24hrs ($p=0.0465$, 95% C.I.=[0.01804, 2.880]. On the other hand, there was no statistically significant difference in mean temperatures between Baseline and 6hrs or between 6hrs ($p=0.8678$) and 24 hrs ($p=0.1010$). The effects of sepsis on temperature were thus seen only in the duration of the 24hrs of the experiment.

A one-way ANOVA for P-Die mice disclosed that there was a statistically significant difference in mean temperature between at least 2 time points ($F(2, 52)=[10.65]$, $p=0.0001$). Tukey's HSD Test for multiple comparisons found that the mean value of temperature was significantly different between Baseline and 6hrs ($p=0.0025$, 95% C.I.=[1.375, 7.318]). There was also a significant difference between Baseline and 24 hrs ($p=0.0002$, 95% C.I.=[2.342, 8.285]). There was no statistically significant difference in mean temperatures between 6hrs and 24 hrs ($p=0.9667$). This demonstrated that the P-Die mice were more susceptible to lower temperatures within 6hrs of CLP induction.

Heart Rate

Figure 4 displays the recorded heart rate (HR) and p-values associated between P-Live and P-Die groups at each time period. Unpaired t tests were performed to compare heart rate in P-Live and P-Die mice.

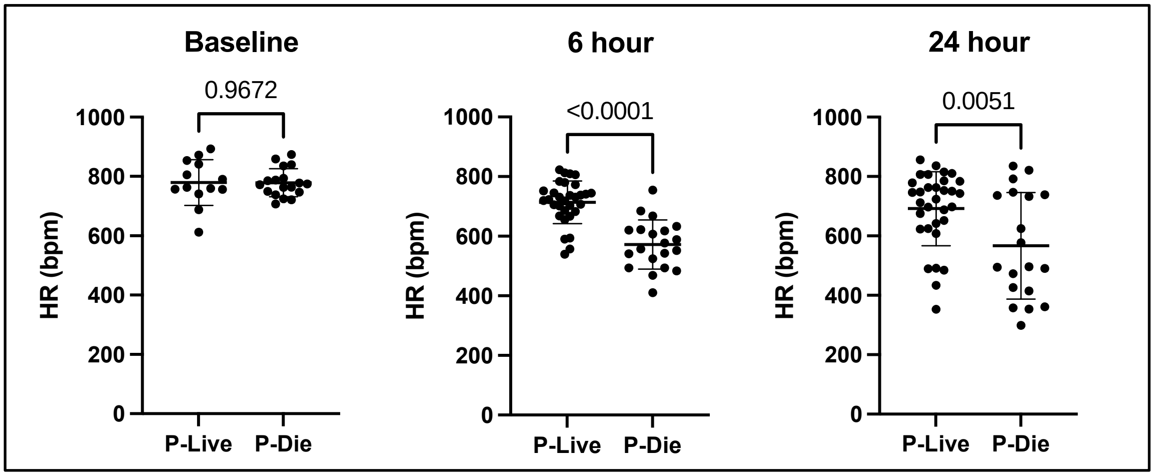

Figure 4. Heart Rate

There was a significant difference in HR 6hrs post-CLP between P-Live (M=713.8, SD=71.4) and P-Die (M=572.1, SD=82.3); t(49)=6.515, p<0.0001. There was also a significant difference in HR 24hrs post-CLP between P-Live (M=691.9, SD=124.7) and P-Die (M=566.9, SD=179.5); t(49)=2.932, p=0.0051. The HR of P-Live mice were significantly higher than that of P-Live mice throughout the duration of the experiment.

One-way ANOVAs with multiple comparisons that were performed to compare the effect of 3 different time points on HR revealed that for P-Live mice there was a statistically significant difference in mean HR between at least 2 time points (F(2, 73)=[3.670], p =0.0303). Tukey's HSD Test for multiple comparisons unveiled a significant difference in the mean value of HR between Baseline and 24hrs (p=0.0228, 95% C.I.=[10.12, 164.9]. There was no statistically significant difference in mean HR between Baseline and 6hrs or between 6hrs ($p=0.1150$) and 24 hrs ($p=0.6517$). It can be recognized that P-Live mice did not experience a strong effect from sepsis during the first 6hrs after CLP. Furthermore, the HR obtained during this time point was when the categorization of P-Live and P-Die mice was determined.

A one-way ANOVA for P-Die mice revealed that there was a statistically significant difference in mean HR between at least 2 time points ($F(2, 54)=[19.43]$, $p<0.0001$). Tukey's HSD Test for multiple comparisons found that the mean value of HR was significantly different between Baseline and 6hrs ($p<0.0001$, 95% C.I.=[114.2, 298.4]). There was also a significant difference between Baseline and 24 hrs ($p<0.0001$, 95% C.I.=[118.4, 304.8]). There was no statistically significant difference in mean HR between 6hrs and 24 hrs ($p=0.9892$). The HR of P-Die mice was strongly affected within the first 6hrs post-CLP.

Respiratory Rate

Figure 5 shows the recorded respiratory rate (RR) and p-values associated between P-Live and P-Die groups at each time period. Unpaired t tests were performed to compare RR in P-Live and P-Die mice. There was a significant difference in RR 6hrs post-CLP between P-Live ($M=181.8$, $SD=26.5$) and P-Die ($M=146.9$, $SD=29.7$); $t(49)=4.373$, $p<0.0001$. The P-Die mice had a significantly lower RR as compared to that of P-Live mice. It is also important to note that this parameter, as aforementioned, had slightly increased between 6hrs and 24hrs post-CLP.

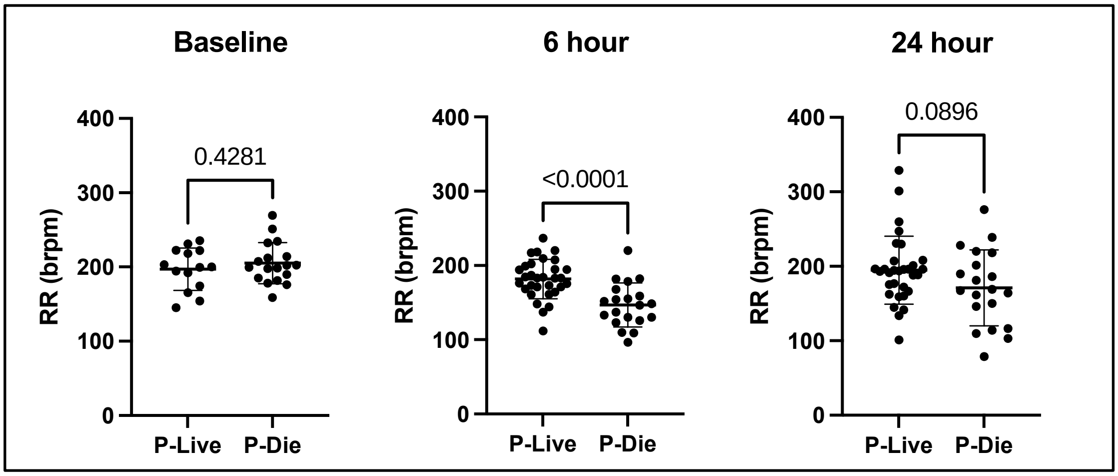

Figure 5. Respiratory Rate

Subsequently, one-way ANOVAs with multiple comparisons were performed to compare the effect of 3 different time points on RR. A one-way ANOVA for P-Live mice revealed that there was no statistically significant difference in mean RR between at least 2 time points (F(2, 73)=[1.359], p =0.2634). A one-way ANOVA for P-Die mice revealed that there was a statistically significant difference in mean RR between at least 2 time points (F(2, 55)=[11.16], p <0.0001). Tukey's HSD Test for multiple comparisons found that the mean value of RR was significantly different between Baseline and 6hrs (p<0.0001, 95% C.I.=[28.42, 87.82]). There was also a significant difference between Baseline and 24 hrs (p=0.0211, 95% C.I.=[4.329, 63.73]). There was no statistically significant difference in mean RR between 6hrs and 24 hrs (p=0.1200). There was an overall decrease in mean RR among P-Die mice.

Oxygen Saturation

Figure 6 shows the recorded oxygen saturation (O2 sat) and p-values associated between P-Live and P-Die groups at each time period.

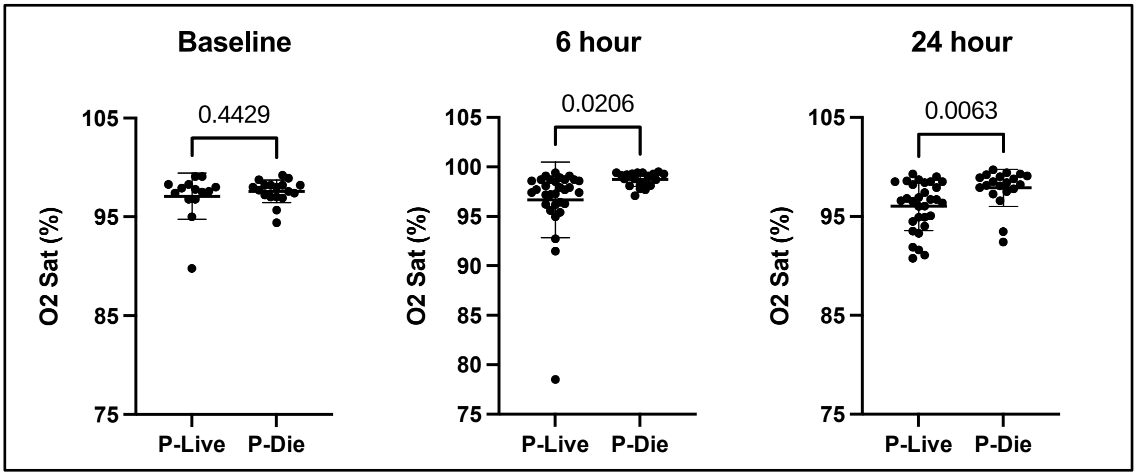

Figure 6. Oxygen Saturation

Unpaired t tests were performed to compare O2 sat in P-Live and P-Die mice. There was a significant difference in O2 sat 6hrs post-CLP between P-Live (M=96.7, SD=3.8) and P-Die (M=98.7, SD=0.7); t(49)=2.392, p=0.0206. There was also a significant difference in O2 sat 24hrs post-CLP between P-Live (M=96.0, SD=2.5) and P-Die (M=97.9, SD=1.9); t(49)=2.852, p=0.0063. During both time points, the O2 sat of P-Die mice were significantly higher when compared to that of P-Live mice.

Furthermore, one-way ANOVAs with multiple comparisons were performed to compare the effect of 3 different time points on O2 sat. A one-way ANOVA for P-Live mice revealed that there was no statistically significant difference in mean O2 sat between at least 2 time points (F(2, 73)=[0.6561], p =0.5219). P-Live mice experienced no change in their mean O2 sat.

A one-way ANOVA for P-Die mice revealed that there was a statistically significant difference in mean O2 sat between at least 2 time points (F(2, 55)=[3.847], p=0.0273). Tukey's HSD Test for multiple comparisons found that the mean value of O2 sat was significantly different between Baseline and 6hrs (p=0.0284, 95% C.I.=[-2.198, -0.1020]). There was no statistically significant difference in mean HR between Baseline and 24hrs (p=0.7784) and between 6hrs and 24 hrs (p=0.1167). Therefore, it can be determined that mean O2 sat was primarily affected within 6hrs post-CLP.

Pulse Distention

Figure 7 exhibits the recorded pulse distention (PD) and p-values associated between P-Live and P-Die groups at each time period.

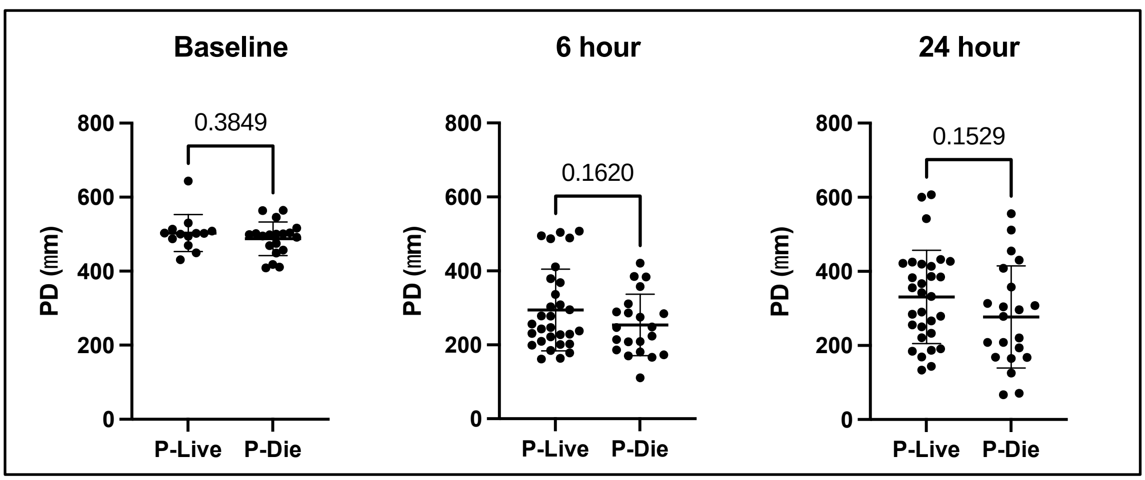

Figure 7. Pulse Distention

Unpaired t tests were performed to compare PD in P-Live and P-Die mice. There was no significant difference in PD between at all time comparisons, though the mean of the mean PD of P-Die mice tended to be slightly lower than that of P-Live mice.

Additionally, one-way ANOVAs with multiple comparisons were performed to compare the effect of 3 different time points on PD. A one-way ANOVA for P-Live mice revealed that there was a statistically significant difference in mean PD between at least 2 time points ($F(2, 70)=[16.77]$, $p <0.0001$. Tukey's HSD Test for multiple comparisons found that the mean value of PD was significantly different between Baseline and 6hrs ($p<0.0001$, 95% C.I.=[120.9, 295.8]). There was also a significant difference between Baseline and 24 hrs ($p<0.0001$, 95% C.I.=[84.29, 259.3]). There was no statistically significant difference in mean PD between 6hrs and 24 hrs ($p=0.4067$). Mean PD was affected in P-Live mice throughout the experiment.

A one-way ANOVA for P-Die mice revealed that there was a statistically significant difference in mean PD between at least 2 time points ($F(2, 58)=[34.12]$, $p <0.0001$). Tukey's HSD Test for multiple comparisons found that the mean value of PD was significantly different between Baseline and 6hrs ($p<0.0001$, 95% C.I.=[159.4, 308.4]). There was also a significant difference between Baseline and 24 hrs ($p<0.0001$, 95% C.I.=[136.5, 285.4]). There was no statistically significant difference in mean RR between 6hrs and 24 hrs ($p=0.7284$). This finding is similar to the mean PD in P-Live mice, which could explain for the lack of significance between mean PD of the two groups at singular points.

Prothrombin (PT)

The PT time for all 81 mice had a median 18.8 (14.15, 114.6). However, the sample size was adjusted by removing mice with PT greater than 50s due to the presence of possible artifact or hemolysis. This resulted in 57 mice with a median 15.5 (12.75, 19.85). The mice were further stratified into two phenotype groups, naïve and sepsis, as seen in Figure 8. The 6 naïve mice had a median of 12.6 (11.2, 15.05); the 51 septic mice a higher median 16.4 (13.3, 22.5). An unpaired t test was performed to compare PT in naïve and septic mice. There was no significant difference in naïve (M=12.93, SD=1.939) and sepsis (M=18.98, SD=8.319); t(55)=1.762, p=0.0837.

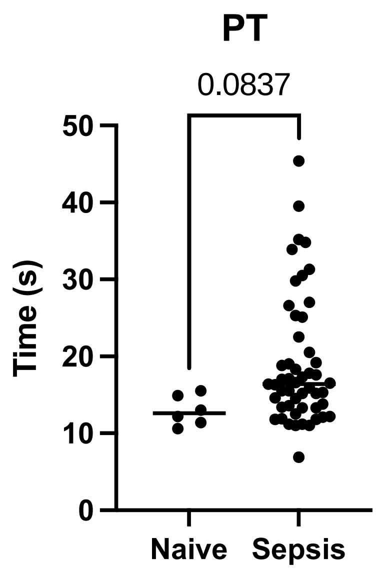

Figure 8. Naïve vs CLP-Induced Sepsis

Another stratification was taken – this time by the type of treatment (HAT/VEH) administered 6hrs post-CLP. This resulted in the exclusion of 11 mice due to incomplete data collection. These four distinct categories are shown in Figure 9.

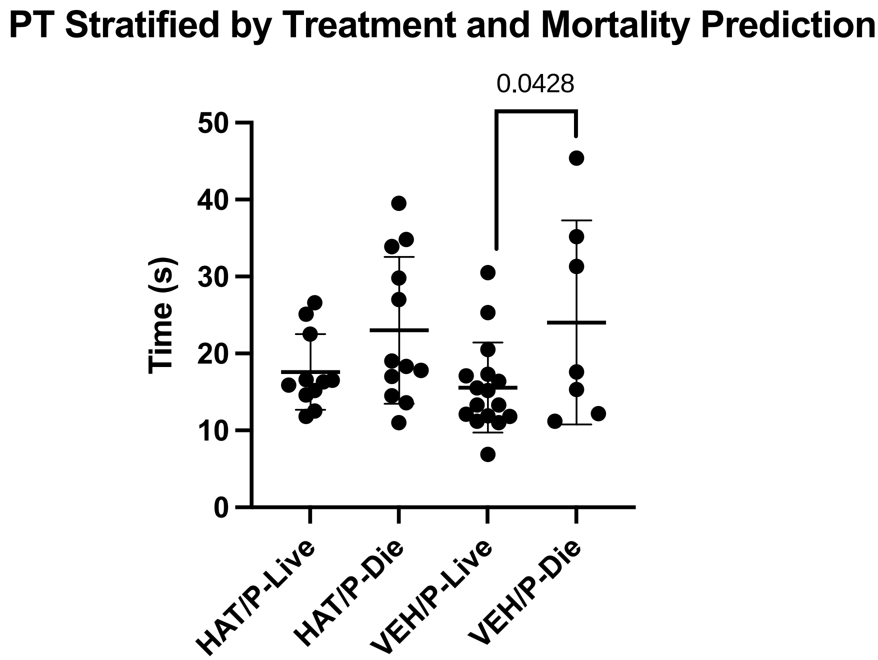

Figure 9. HAT vs VEH Treatment

Unpaired t tests were performed to compare PT in HAT/P-Live, HAT/P-Die, VEH/P-Live, and VEH/P-Die groups. There was a significant difference in PT between VEH/P-Live (M=15.58, SD=5.8) and VEH-P-Die (M=24.03, SD=133); t(2)=2.156, p=0.0428. However, there were no significant differences between the means of VEH/P-Live and HAT/P-Live (p=0.3575), the means of HAT/P-Live and HAT/P-Die (p=0.1602), nor the means of HAT/P-Die and VEH/P-Die (p=0.8488).

In comparisons via a one-way ANOVAs, the results revealed that there was a statistically significant difference in mean PT between at least 2 time points (F(3, 42)=[2.866], p=0.0478). However, Tukey's HSD Test for multiple comparisons revealed no significant pairwise differences due to the unequal sample sizes per group. Table 3 gives a more in-depth report of the descriptive statistics of PT stratified by 4 categories based on treatment and mortality predictions.

Table 3. Descriptive statistics of the PT stratified by 4 categories based on treatment and mortality predictions

	HAT/P-Live	HAT/P-Die	VEH/P-Live	VEH/P-Die
n (N=46)	11	12	16	7
Minimum	11.8	11.0	6.9	11.2
25% Percentile	14.6	15.1	11.8	12.2
Median	16.3	18.7	14.3	17.6
75% Percentile	22.5	32.9	17.3	35.2
Maximum	22.6	39.5	30.5	45.4
Mean	17.6	23.0	15.6	24.0
Std. Deviation	4.9	9.5	5.8	13.3
Std. Error of Mean	1.5	2.8	1.5	5.0
Lower 95% of CI of mean	14.3	17.0	12.5	11.8
Upper 95% CI of mean	20.9	29.1	18.7	36.3

GPC

Results of GPC-1

After the mice were sacrificed, the collected plasma samples were used to test for prothrombin time as well as the presence of glypicans (GPC). The first ELISA performed was to determine the presence and levels of GPC-1 in the plasma of both CLP-induced septic mice and naïve mice. The results are shown below in Figure 10.

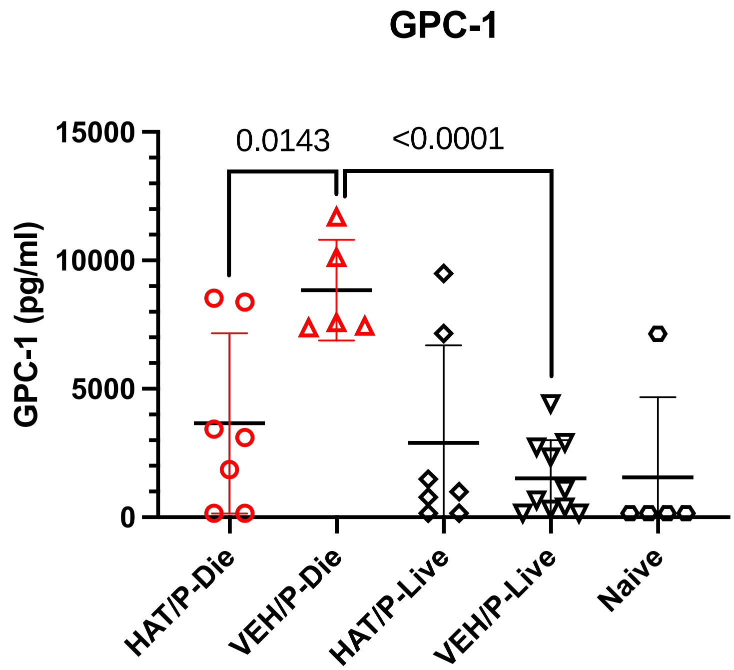

Figure 10. Concentration of GPC-1. Graph credits to Jiyoun Kim, Ph.D.

An unpaired t test was performed to compare GPC-1 in HAT/P-Live, HAT/P-Die, VEH/P-Live, and VEH/P-Die groups. There was a significant difference in GPC-1 level between HAT/P-Die (M=3658, SD=3516) and VEH/P-Die (M=8845, SD=1966); t(1)=2.959, p=0.0143. In other words, HAT therapy had an effect on GPC-1 in mice predicted to die. There also a significant different in GPC-1 between VEH/P-Live (M=1516, SD=1485) and VEH/P-Die (M=8845, SD=1966); t(1)=8.120, p<0.0001. GPC-1 levels in P-Die mice were higher than that in P-Live mice among the VEH group. On the other hand, there were no significant differences between the means VEH/P-Live and HAT/P-Live (p=0.3125) nor the means of HAT/P-Live and HAT/P-Die (p=0.7014).

For further analysis, one-way ANOVAs with multiple comparisons were performed to compare the effect of treatment and mortality prediction on GPC-1. A one-way ANOVA for HAT/P-Die, VEH/P-Die, and VEH/P-Live mice revealed that there was a statistically significant difference in mean GPC-1 between at least 2 time points (F(2, 19)=[15.58], p <0.0001. Dunnett's Test for multiple comparisons found that the mean value of GPC-1 was significantly different between VEH/P-Die and HAT/P-Die (p=0.0029, 95% C.I.=[1861, 8512]). There was also a significant difference between VEH/P-Die and VEH/P-Live (p<0.0001, 95% C.I.=[4218, 10440]).

Results of GPC-4

The presence of GPC-4 was also investigated in another ELISA experiment. In literature, both GPC-1 and GPC-4 were commonly studied together. The results are shown below in Figure 11.

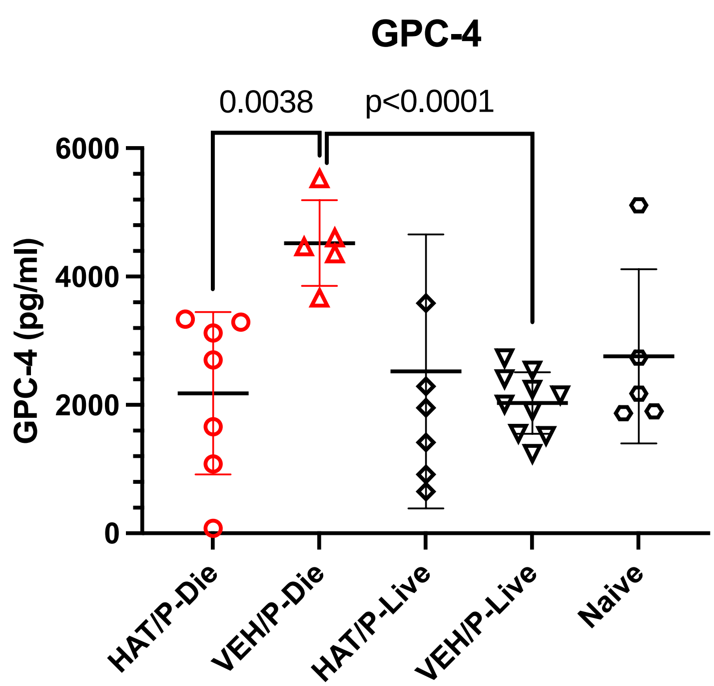

Figure 11. Concentration of GPC-4. Graph credits to Jiyoun Kim, Ph.D.

An unpaired t test was performed to compare GPC-4 in HAT/P-Live, HAT/P-Die, VEH/P-Live, and VEH/P-Die groups. There was a significant difference in GPC-4 level between HAT/P-Die ($M=2182$, $SD=1267$) and VEH/P-Die ($M=4525$, $SD=667.3$); $t(1)=3.746$, $p=0.0038$. This demonstrated that HAT had effect on GPC-4 levels in P-Die mice. There was also a significant difference in GPC-4 between VEH/P-Die ($M=4525$, $SD=667.3$) and VEH/P-Live ($M=2033$, $SD=480.3$); $t(13)=8.351$, $p<0.0001$. P-Die mice presented with elevated GPC-4 levels compared to P-Live mice when receiving VEH treatment. The last finding from the unpaired t tests was that there were no significant differences between the means VEH/P-Live and HAT/P-Live ($p=0.4893$) nor the means of HAT/P-Live and HAT/P-Die ($p=0.7232$).

To compare the effect of treatment and mortality prediction on GPC-4, one-way ANOVAs with multiple comparisons were performed. A one-way ANOVA for HAT/P-Die, VEH/P-Die, and VEH/P-Live mice revealed that there was a statistically significant difference in mean GPC-4 between at least 2 time points ($F(2, 19)=[16.14]$, $p<0.0001$. Dunnett's Test for multiple comparisons found that the mean value of GPC-4 was significantly different between VEH/P-Die and HAT/P-Die ($p=0.0003$, 95% C.I.=[1175, 3510]). There was also a significant difference between VEH/P-Die and VEH/P-Live ($p<0.0001$, 95% C.I.=[1400, 3584]).

Relationship between GPC-1 and GPC-4

Having attained results from ELISAs run for GPC-1 and GPC-4, further analysis was necessary to determine if there was a relationship between the two glypican levels as portrayed in previous literature. Thus, a simple linear regression was used to test if GPC-1 levels significantly predicted GPC-4 levels. This can be seen in Figure 12.

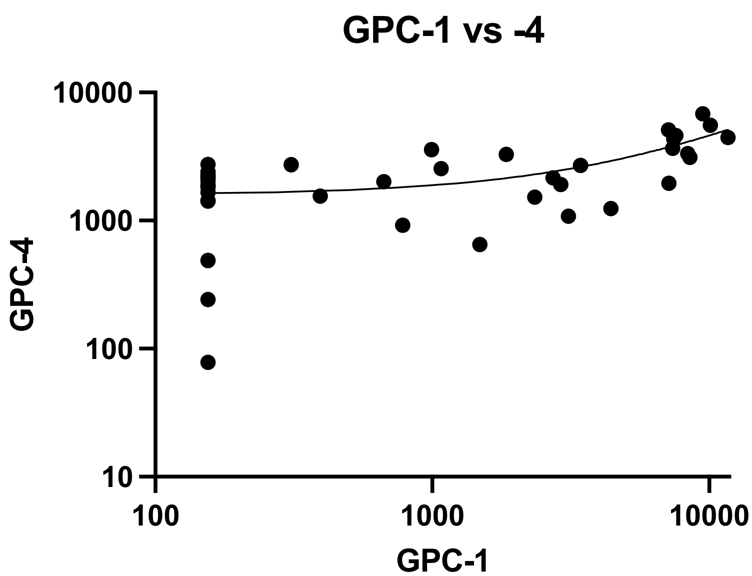

Figure 12. GPC-1 vs GPC-4. Graph credits to Jiyoun Kim, Ph.D.

The fitted regression model was y=0.3026*x +1585. The overall regression was statistically significant (R^2=0.5404, F(1, 38)=44.68, p<0.0001). Therefore, it was found that GPC-1 significantly predicted GPC-4 levels. Furthermore, a Pearson correlation coefficient was computed to access the linear relationship between GPC-1 and GPC-4. This analysis determined that there was a positive correlation between the two variables r(40)=.74, p<0.0001. With elevated GPC-1 levels, increasing levels of GPC-4 can also be observed in the plasma of the CLP-induced sepsis murine models. This finding is comparable to that of previous studies.

DISCUSSION

The research experiments of this study were premised on the finding from a small clinical trial that hydrocortisone, ascorbic acid, and thiamine (HAT) therapy improves survival in sepsis (Marik et al., 2017). The murine model of sepsis was used to replicate the clinical trials and determine if the same results could be reproduced. If so, the animal model could be used in further examinations for potential mechanisms in treating sepsis.

Physiological Parameters

To increase the strength of the experimental design, the physiologic measurements were used to stratify mice into those predicted to live and those predicted to die. In previous studies, mice with a lower HR below 620bpm were found to have significantly greater mortality when compared to mice with a HR >620bpm 6hrs post-CLP (Kim et al., 2020). Other discrimination values included PD of 300 μm and RR 150brpm, but were not found to play a significant role in mortality prediction (Kim et al., 2020).

Past clinical studies have shown that sepsis mortality can be predicted by early myocardial dysfunction (Havaldar, 2018; Kim et al., 2020; Rolando et al., 2015). In septic patients, myocardial dysfunction along with myocardial depression occur in almost 40-50% of patients – it is a recognized manifestation of sepsis (Khan et al., 2013; Rudiger & Singer, 2007). Contractile dysfunction can be characterized by " biventricular dilatation, blunted blood pressure response to intravenous fluids, a reversible reduction in ejection fraction (EF) and a diminished ability to augment cardiac output despite increased levels of circulating catecholamines" (Flynn et al., 2010; Khan et al., 2013). On the other hand, cardiac depression after CLP-induced sepsis has been demonstrated to present with a reduction in the EF within 24hrs and decreases in HR (Khan et al., 2013; Zhou et al., 2017). One study found CLP- induced sepsis significantly impaired cardiac function within 6hrs (Ma et al., 2016). This finding was reproduced in this current experiment in which significant differences in HR were found to take place within 6hrs post-CLP. In conclusion, it can be inferred that quick intervention targeting the 6hr window from the introduction of sepsis can be crucial in improving health outcomes in mice. For further research investigation, it is important to factor in that heart rate in septic mice decreases, while it is elevated in humans. Thus, translational research should be cautious of this distinction.

P-Die Mice Have Increased PT

In the clinical setting, sepsis-associated coagulopathy are typically diagnosed by prolonged prothrombin or an elevated international normalized ratio, and reduced platelet count (Walborn et al., 2018). This experiment determined that there was no significant difference of PT between naïve and septic mice though septic mice tended to have increased PT. However, when the septic mice were further stratified by treatment and mortality prediction phenotypes, a significant difference was found between VEH/P-Live and VEH/P-Die. Prothrombin was markedly increased in plasma levels of VEH/P-Die mice at 24hrs. It is important to note that this change can primarily be seen in mice with a poor mortality prediction (P-Die) and independent of therapy received. This also confirms that murine models can be representative of the human coagulation disturbances in sepsis. This finding is similar to another experiment that found that VEH treatment consisting solely of imipenem reduced the CLP-evoked decrease in thrombin generation and consumption of prothrombin 24hrs post-CLP (Wang et al., 2014). No conclusive evidence could be drawn on the effects of HAT treatment on PT times from this experiment.

Sepsis Elevates GPC in P-Die Mice

The shedding of the endothelial glycocalyx is gradually being recognized as an important mechanism of sepsis. However, it is currently unclear if glypicans directly contribute or have a role in this response (Fisher et al., 2019). The results of the statistical analysis comparing levels of GPC-1 and GPC-4 among 4 different sepsis phenotype groups of the study showed two significant pairwise differences. The significant difference between VEH/P-Live and VEH/P-Die Mice show that GPC-1 and 4 are elevated in the plasma of sepsis patients and indicates that these glypican components of the glycocalyx may be involved in the pathophysiological mechanism of sepsis (Fisher et al., 2019). There was not a significant difference in mean levels with naïve mice, thus it was inconclusive if GPC-1 and GPC-4 are generally elevated in septic mice.

HAT Reduces GPC levels

The second significant pairwise differences in the mean GPC-1 and 4 levels were between HAT/P-Die and VEH/P-Die, which demonstrates that HAT attenuates levels of glypicans in the plasma. It can be concluded that HAT improves the outcomes of only mice predicted to die because there was no significant difference in GPC-1 levels between HAT and VEH treated P-Live mice. A possible rationale for this finding lies in the effects of hydrocortisone, which is one of the components of HAT therapy. A study in

2007 discovered that hydrocortisone had protective effects on the vascular endothelial glycocalyx during an experiment of ischemia-reperfusion injury of isolated guinea pig hearts (Chappell et al., 2007). This finding can lead to potential studies looking into this mechanism of hydrocortisone therapy and the decreased shedding of the glycocalyx that is typically seen in sepsis.

Limitations

The primary limitation of this study came from errors in data collection, which led to incomplete data from the noninvasive physiological measurements. Data analysis of stratification of mice by treatment type was unable to be accomplished due to various confounding variables. This issue also led to an exclusion of mice during analysis, decreasing the power of the study. Thus, more experiments must be run to increase the power, as well as the validity and reliability.

Future Studies

To obtain a complete set of DIC scores requires one other component. While the complete blood count was collected for platelet count in addition to PT, the D-Dimer was not measured. Thus, the extent of this study was a preliminary determination of the effects of CLP-induced sepsis on PT in murine models. Further research can validate the DIC score in septic mice and initiate more studies for translational opportunities.

Previous research demonstrated that sepsis results in the concomitant initiation of the inflammatory and procoagulant pathways that leads to prolonged PT levels (Rittirsch et al., 2009). PT reflects both liver protein synthesis as well as consumption by coagulation activation. Thus, PT is a sensitive marker of the severity of hepatocellular damage, possibly extended with liver cell necrosis or liver cirrhosis (Li et al., 2018). Studies have shown that liver and kidney dysfunction is an important pathological sepsis phenotype (Li et al., 2018). In this experiment, CLP may directly affect liver function. Therefore, looking into this dynamic through means such as measuring liver synthesis independent of PT would provide more insight into the mechanisms of sepsis and its multiorgan effects.

Previous studies have seen an association of GPC-1, GPC-3, and GPC-4 with the development of organ failure. It was demonstrated that plasma GPC-3 and GPC-4 (but not GPC-1) were significantly elevated in patients who developed organ failure as compared to the control group consisting of patients who had never developed organ failure (Fisher et al., 2019). Further studies on GPC levels should determine that the levels are not changing due to clearance. This will also require an analysis assessing the organ quality and severity of septic mice.

CONCLUSION

In conclusion, the study found mice predicted to die to have lower physiologic parameters, prolonged prothrombin times, and elevated plasma glypican levels. These confirm previous studies and literature regarding the coagulation cascade, possible organ dysfunction with the breakdown of the endothelial membrane, as well as the pathogenesis of sepsis. In the future with more research investments, it can be rewarding to further investigate the pathophysiology of sepsis and figure out the potential treatment and therapy to mitigate this overwhelming public health problem. Furthermore, data is limited surrounding the small study around using HAT therapy, and so it will be interesting to see if there are new discoveries of potential therapies targeting the same mechanism or different mechanisms of sepsis.

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