Physiologic Effects of Low-Level Nutritional Support in a Mouse Model of *Klebsiella pneumoniae* Sepsis

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Abstract

Sepsis is an illness characterized by high morbidity and mortality, accounting for 1 in 3 hospital deaths, affecting 1 - 3 million adults in the US every year. Among sepsis cases, approximately 12% are complicated by the incidence of hyperglycemia, a condition that is associated with worsened outcomes including higher mortality. Prior work in our lab has shown the association between the provision of early enteral nutritional support with attenuated inflammatory responses, preservation of euglycemia through heightened insulin sensitivity, and improved mean arterial blood pressure in a mouse model of LPS-induced endotoxemia. As this is a sterile and some would argue, unrealistic model of sepsis, the aim of this study was to see if these results could be observed in a bacteremic model of sepsis, with a focus on mortality as one of the primary outcomes.

Ten week old male B6 mice were infected with *K. pneumoniae* (strain ATCC 43816 $2x10^4$ CFUs) via oropharyngeal aspiration after implantation of catheters in the femoral artery and femoral vein or stomach. 24hr. post infection, mice were started on low-level infusions of either intravenous dextrose, enteral dextrose, or enteral saline. Blood was sampled at 24hr. intervals. Mice were sacrificed when blood pressure fell below 61mmHg for at least an hour, or at 72hr. post infection. BAL and tissue samples were then collected.

At 72hr., mice receiving enteral dextrose had a mortality rate of 27%, compared to 78% in the enteral saline group and 100% in the IV dextrose group. Mice receiving enteral dextrose also

maintained euglycemia and cardiovascular homeostasis for the duration of the experiment, compared to IV dextrose mice who became hyperglycemic and hypotensive and enteral saline mice who trended towards hypoglycemia and hypotension. Metrics of inflammation and lung injury were also significantly different between groups in some outcomes.

The results of this study suggest the possible therapeutic benefits of early low-level nutritional support in cases of sepsis. Of public health significance, enteral dextrose therapy could be used to decrease the incidence and severity of post-sepsis complication development, specifically loss of muscular and neurological function. It is possible that this is clinically relevant and could have implications for treatment strategies in the future for sepsis.

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Preface

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1.0 Introduction

1.1 Sepsis Is Common, Costly, and Often Lethal

Sepsis is a significant global public health concern with high incidence, severe mortality and morbidity rates, and heavy economic burden. While methodologies for diagnosis are variable, recent analyses of the global incidence estimate rates as high as 50 million/yr (1). In critically-ill patients, sepsis is estimated to have an in-hospital mortality rate of up to 35% (2, 3), and this statistic does not take into account post-hospitalization mortality, nor does this statistic fully convey the severity of the effects of long-term sepsis-related outcomes. In the US, sepsis accounts for approximately 5% of all annual health costs, totaling about $20 billion/yr (4). Survivors of sepsis experience lower earning potential, higher chance of joblessness, and decreased coverage by private health insurance following discharge (5). The economic costs of long-term sepsis-related complications are only now being studied. It is important to note that many of the statistics on sepsis are taken from studies in developed countries. Given the significant association between higher mortality rate and lower gross national income, sepsis likely poses an even larger problem in developing countries (1).

Within the last decade, the incidence rate of sepsis has been steadily climbing, likely due to increased awareness, better reporting, and a larger aging population that is more likely to develop sepsis due to other comorbidities (6). In the US, 3-year and 5-year survival has doubled in the last two decades in patients over 65 years of age (6). Unfortunately, both cognitive impairment and functional disability, collectively termed post-intensive care syndrome (PICS), have been reported as consequences of surviving sepsis, of which an estimated 20,000 new

cases/yr. of moderate to severe cognitive impairment are attributed. Sepsis is associated with the development of many functional limitations in elderly patients including difficulty preparing meals, using the phone, and getting dressed (7). Quantifying the additional unmeasured burdens of sepsis (increased caregiver time, incidence of depression, family burden) is difficult, but it is clear that sepsis represents a complex challenge from a public health, as well as an individual healthcare perspective.

1.2 Sepsis is a Complex Disease Defined by the Host Response

The complexity of sepsis is also evident in how it is defined. The current definition of sepsis comes from the Third International Consensus Definitions for Sepsis and Septic Shock, which states that sepsis is "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (8). A dysregulated host response is a pathogenic inflammatory response in which high levels of pro-inflammatory cytokine, chemokine, and other immune cell product e.g. reactive oxygen species activity leads to excessive vasodilation, cellular dysfunction, and metabolic complications. A decrease in systemic blood pressure is a hallmark of septic shock, and ultimately leads to multiple-organ failure (presumably from inadequate blood perfusion to vital organs) and death (8). Several studies have shown IL-6 as a pro-inflammatory cytokine of importance in sepsis prognosis. IL-6 is secreted, mainly by macrophages, downstream of Toll-like receptor (TLR) signaling in response to pathogen-associated molecular pattern molecules (PAMPs). For example, in gram-negative bacterial sepsis, the innate immune response is triggered through TLR4 recognition of the PAMP lipopolysaccharide (LPS) on the bacterial cell surface. Baseline IL-6 levels in septic patients can predict risk of organ failure and mortality, along with

other cytokines, including IL-1β and anti-inflammatory cytokine IL-10, that are significantly elevated in sepsis (9, 10). TNF-α has also been studied as a primary mediator of the septic inflammatory response and is secreted mainly by macrophages. TNF-α is released concurrently with IL-1β in response to TLR signaling (11, 12). Given TNF-α's role in recruiting neutrophils, increasing endothelial wall adhesion, and upregulating inflammatory lipid factor production, it is necessarily associated with increased intravascular coagulation, hypotension, and organ failure in sepsis (13, 14). In general, current understanding of sepsis pathobiology implicates the innate immune system as the pathogenic source, though several nonimmunologic pathways in cardiovascular, endocrine, neuronal, and metabolic systems, amongst others, are also involved.

Due to the multitude of factors involved, one of the main challenges in sepsis is establishing standard criteria for recognition and diagnosis (8). There is no gold standard diagnostic test; diagnosis is based on clinical signs and symptoms. The most recent criteria focus on identifying early organ dysfunction as well as establishing presence of an infection. A Sequential Organ Failure Assessment (SOFA) score is assigned based on a variety of prognostic markers from several systems, where an acute increase of ≥2 serves as a proxy for organ dysfunction (Table 1). Septic shock is defined as sepsis accompanied by a persistent hypotension (defined as mean arterial blood pressure ≤ 65mmHg) unaffected by fluid resuscitation (8). Whereas recognition and consensus diagnosis of sepsis has improved, the complex nature of sepsis makes treatment especially difficult.

Table 1. Sequential Organ Failure Assessment Table

	Score				
System	0	1	2	3	4
Respiration					
PaO_2/FiO_2, mmHg	≥400	<400	<300	<200 with respiratory support	<100 with respiratory support
Coagulation					
Platelets, /μL	≥150,000	<150,000	<100,000	<50,000	<20,000
Liver					
Bilirubin, mg/dl	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12.0
Cardiovascular					
MAP and adrenergic agents	MAP≥70 mmHg	MAP<70mmHg	Dopamine or dobutamine (any dose)	Epinephrine or norepinephrine (any dose)	-
Central nervous system					
Glasgow Coma Scale score	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL	<1.2	1.2-1.9	2.0-3.4	3.5-4.9	>5.0
Urine output, mL/d				<500	<200

SOFA scores characterize organ failure in the respiratory, cardiovascular, neuronal, hematologic, hepatic, and renal systems (15).

Treatment for sepsis is complicated by the heterogeneity inherent in both affected individuals and healthcare settings: age, comorbidities, previous medical interventions such as drug use or surgery, and nature of the infectious agent must all be considered. Management strategies usually default to treating general symptoms. Intravenous (IV) fluid administration is provided early after diagnosis to manage hypotension. When septic shock occurs, anti-hypotensive agents known as vasopressors are used to induce vasoconstriction and raise blood pressure. If the infectious agent is bacterial, appropriate antibiotics are given once the pathogen is identified. Sepsis may have a severe impact on the lungs, resulting in the development of Acute Respiratory Distress Syndrome (ARDS) (16); oxygen may be administered or, if hypoxemia is severe, patients are placed on a ventilator (8). These treatment options address the general and acute symptoms associated with sepsis, but there is another side to the disease that must be considered.

1.3 Sepsis has Profound Effects on Metabolism

Sepsis triggers a severe catabolic state, where muscle, fat, and glycogen stores are rapidly broken down for glucose production (17). Catabolic patients experience rapid muscle atrophy and weakening in skeletal muscle, leading to a higher chance of developing thromboembolisms and ICU-acquired weakness (ICU-AW), and exacerbating pulmonary complications and possibly increasing time on ventilators (18, 19). Muscle weakening is further complicated by sepsis-induced polyneuropathy, characterized by blunted motor and sensory neuron excitability, and bioenergetic failure. The resulting effect of this combination of symptoms is even greater weakness than would occur with muscle atrophy alone (20, 21). ICU-AW complicates in-hospital recovery and is associated with higher long-term mortality after discharge (22). The impact of ICU-AW and PICS highlight the need for effective treatment options during a septic patient's ICU stay. Current research has focused on two methods, nutritional/caloric support and hormone supplementation.

Multiple studies focusing on nutrition and sepsis have shown a survival and quality of life benefit when patients are provided with adequate caloric provisions. Prospective trials measuring acute care (e.g. ventilator-free days) and long-term outcomes (e.g. multi-month/year post-sepsis survival and physical ability) found positive associations with increased caloric support (23-25). However, these results were complicated by confounding variables including patient body mass index and increases in patient survival, and physical function became less significant at longer follow-up times. Moreover, not all trials were able to reproduce beneficial effects. Notably, the EDEN trial showed no difference in clinical outcomes between trophic (low-level) and full feeds, aside from fewer episodes of more acute gastrointestinal intolerance (26, 27). Another point of discussion on the value of nutrition in sepsis is the issue of route. Specifically, septic patients can receive enteral nutrition, parenteral/intravenous nutrition (IVN), or both; there are published

guidelines and advocates for both methods, and no clear standard of practice (28-30). The issue of nutrition administration route will be discussed in detail in related topics later in this Introduction. Although it would seem intuitive that caloric provision is the most straightforward strategy for countering a catabolic state in sepsis, no consensus exists on how to optimize the approach.

Anabolic hormones have been explored as an alternative to treating catabolism during sepsis, with insulin being one of the most promising options. Insulin exerts protein synthesis stimulating effects *in vivo* and is associated with reduced protein degradation (31, 32). However, insulin as a treatment requires not only a discussion of catabolism, but also of glucose regulation as dysglycemia is commonly seen in sepsis.

1.4 Dysglycemia Complicates Outcomes in Sepsis

Hyperglycemia is observed in 12% of all sepsis cases in the US and is associated with increased mortality and morbidity (33-35). The rise in blood glucose (BG) in response to illness/injury has been termed "stress hyperglycemia" and is triggered by excessive activity of counterregulatory hormones such as glucagon, growth hormone, glucocorticoids, high levels of pro-inflammatory cytokines (particularly TNF-α), and a relative insulin deficiency (36, 37). Hyperglycemia is further complicated by uninhibited hepatic gluconeogenesis and impaired glucose uptake by skeletal muscle due to systemic insulin resistance, the latter commonly seen in sepsis independent of hyperglycemia (38, 39). Stress hyperglycemia is a distinct hyperglycemic phenotype; whereas significant increases in mortality and morbidity are associated with stress hyperglycemia, similar outcomes are not observed in septic patients with diabetic hyperglycemia,

despite similar BG levels (40). To address stress hyperglycemia in sepsis, trials were started in the early 2000s exploring the use of intensive insulin therapy (IIT) to manage BG.

The 2001 IIT clinical trial conducted by Van den Berghe et al. (41) was one of the most influential studies conducted in the field of sepsis treatment. Patients in a surgical ICU were randomized to receive either IIT (insulin administration to keep BG 80-100mg/dL) or conventional insulin treatment (insulin given only when BG >215mg/dL). Use of IIT resulted in an absolute risk reduction in mortality from 8.0% to 4.6% between the two groups and was also associated with significant reductions in outcomes of morbidity such as duration of intensive care (only in patients staying >5 days), duration of ventilatory support, incidence of renal failure, bacteremia, and polyneuropathy. The highest mortality benefit was observed in patients with multiple-organ failure, suggesting this therapy was especially effective in the setting of severe sepsis (41). While promising at the time, results of subsequent studies and analyses were unable to reproduce findings from the initial trial. The majority of evidence (see below) pointed towards not only an absence of a survival benefit with IIT, but that use of IIT may actually be causing harm.

Several studies carried out after the Van den Berghe trial expanded the study population from patients in surgical ICUs to both medical and mixed ICUs; subsequent results contradicted those from the initial 2001 study. Together, the new trials suggested that IIT was associated with either no difference or even an increase in mortality/morbidity (42-46). One trend identified in several studies was the association of IIT with increased incidence of hypoglycemia, and subsequent hypoglycemia-associated adverse events. A comprehensive meta-analysis of all randomized trials investigating tight vs. conventional BG control from 2001 – 2008 clarified that IIT is not associated with any mortality/morbidity benefits but is associated with increased incidence of hypoglycemia (47). The 2009 NICE-SUGAR trial, an international multi-center

clinical trial enrolling a total of 6104 patients, also found no significant difference in morbidity-related outcomes, but showed a significant association between IIT and increased mortality and incidence of hypoglycemia (48). The trials highlight the complexity and number of interacting variables at play in the clinical setting. The effects of nutritional support, the route of nutrition, endogenous insulin response to nutrition, exogenous insulin treatment, and metabolic dysregulation and hyperglycemia are all intertwined. Observational studies and trials on heterogeneous patient populations have thus far not provided clarity around treatments and outcomes. A need exists to go back to the controlled laboratory setting and systematically explore the underlying mechanisms by which nutrition and insulin impact sepsis.

1.5 Glucose Regulation in Mouse Models of Sepsis

The 2001 IIT trial and subsequent trials provided rigorous data on BG levels in all aspects of patient treatment (baselines, targets, cutoffs, optimal ranges etc.) but did not place the same emphasis on measuring plasma insulin at baseline or during therapy. In order to tease apart the relationship between insulin, hyperglycemia, and mortality in sepsis, Woodske et. al. (49) conducted a study in an endotoxemic mouse model (LPS; lipopolysaccharides found on the outer cell wall of gram-negative bacteria) with controlled pharmacologic manipulation of circulating insulin using diazoxide. Two important observations were made: 1) LPS exposure was associated with significantly higher circulating insulin levels in mice receiving equal amounts of intravenous dextrose (IVD) compared to healthy controls (with similar BG levels) and, 2) at matched levels of hyperglycemia, survivors had 2 to 3-fold higher circulating insulin levels than non-survivors. These data suggest that insulin may have glucose-independent protective effects in the context of

sepsis, and endogenous insulin insufficiency could be a predictor for mortality. A subsequent study from the same group using both an LPS model as well as a cecal ligation puncture (CLP) model observed that neither IVD nor a septic insult alone was associated with hyperglycemia (50). Rather, the combination of IVD and endotoxemia/sepsis was associated with marked glucose intolerance, insulin resistance, insufficient insulin secretion in response to glucose challenge, and hyperglycemia. In the CLP model, septic mice receiving IVD had 40% mortality at 24hr. compared to 0% in septic mice receiving IV saline and healthy mice receiving IVD. The results showed that insulin and glucose-related complications were driven not by sepsis alone, but by sepsis in combination with early IVD supplementation; a hypothesis reinforced by the fact that parenteral nutrition has been associated with a high incidence of hyperglycemia in critically ill humans (35, 51). The mouse model also showed that plasma insulin did not correlate with BG in moderate or severe hyperglycemia, implying an insufficient pancreatic insulin response as a significant factor in the development of hyperglycemia (50). Interestingly, whereas exogenous insulin administration lowered the incidence of hyperglycemia when given at the time of septic insult, continuous high-level insulin infusion was unable to rescue septic mice if started after hyperglycemia has occurred (52). Overall, the mouse studies suggest that metabolic dysregulation is due to a two-hit effect from both sepsis and early IV nutrition, whereas either alone is not sufficient to produce hyperglycemia. Clinically it could be argued to administer insulin early to prevent hyperglycemia, but such a treatment would be analogous to IIT which had already been shown to increase the risk of hypoglycemia in humans. Therefore, an alternative approach to endogenously stimulate appropriate insulin release early in the progression of sepsis is needed, and it is here that the issue of nutritional provision route becomes relevant.

1.6 The Route of Nutrition Administration in Sepsis

Studies on the effects of nutritional support route in humans have, thus far, been more observational in nature rather than interventional. Most studies in septic patients published in the past two decades have focused on reporting outcomes associated with one route or the other, with no studies directly comparing IV vs. enteral nutrition in one patient cohort. Thus, potential benefits and negative effects have been reported in the use of both routes of delivery. One of the most significant concerns regarding parenteral nutrition (IVN) has been its association with a high incidence of hyperglycemia. Indeed, many observational studies across multiple study populations have established that IVN-associated hyperglycemia is independently associated with increased mortality and risk of hospital complications (35, 51, 53, 54). Nevertheless, parenteral nutrition is still considered effective in preventing malnutrition and reducing complication rates, and thus remains standard practice in some medical fields (55). Van den Berghe showed that when artificially controlling for hyperglycemia, IV glucose could be used to blunt catabolic responses in critically ill rabbits without any adverse effects (56). However, the same group also showed in a subsequent study that early IV glucose provision in critically ill rabbits induced an autophagy-deficient phenotype characterized by dysregulated mitochondrial activity and liver damage (57). Thus, there is evidence in both animal models and clinical populations that parenteral may not be the ideal route for administration of nutrition.

Enteral feeding is not without issues either. Problems associated with the enteral route include tube dislodgement, diarrhea, constipation, nausea/vomiting, pulmonary aspiration, nasal ulcer (feeding is usually done through a nasogastric tube), and whereas the incidence is lower than with IVN, hyperglycemia can still occur (58). However, it is clear that the long-term adverse outcomes such as ICU-AW and PICS need to be managed in sepsis; patients must be fed despite

the risks. To address how best to deliver nutrition in critical illness a pre-clinical study was conducted by Shah et. al. (59) to develop a therapeutic method that could facilitate both caloric and insulin provision in the context of sepsis. In an LPS model, mice were subjected to a glucose tolerance challenge in the context of continuous low-level parenteral or enteral dextrose infusions. Endotoxemic mice receiving parenteral dextrose exhibited significant glucose intolerance, blunted insulin sensitivity, insufficient insulin secretion, and marked insulin resistance, resulting in severe hyperglycemia after dextrose administration that did not resolve. In contrast, endotoxemic mice that received the same amount of dextrose through the enteral route exhibited normal glucose tolerance, insulin sensitivity, and insulin secretion comparable to healthy controls and saline-challenged septic mice. While transiently hyperglycemic immediately following the dextrose challenge, endotoxemic mice receiving enteral dextrose cleared the bolus and returned to euglycemic levels in the same time frame as control mice. In addition to controlling glucose homeostasis there were other noted benefits of enteral nutrition administration in the mouse model of endotoxemia.

Enteral nutrition can both affect inflammatory and activate incretin pathways during sepsis. In the Shah et al. LPS model (59), circulating pro-inflammatory cytokines were significantly lower in enteral dextrose-infused septic mice compared to IVD infused septic mice. The enteral route of nutritional support was also associated with significantly higher circulating levels of a duodenum-secreted insulin-stimulating hormone, glucose-dependent insulinotropic peptide (GIP). GIP is an incretin hormone that is released from the gut to act on pancreatic β-cells and induce insulin secretion. Importantly, the insulin secreting effects of GIP (and other incretins) are modulated by the prevailing glycemic environment—at high glucose levels GIP is very effective at stimulating insulin secretion, but at low glucose levels GIP is relatively ineffectual (60). Together, these results

suggest that early enteral dextrose (ED) administration has potential as a treatment against sepsis through its endogenous stimulation of glucose-dependent insulin release. That is, ED could be used to stimulate insulin secretion in such a way that hypoglycemia associated with IIT could be naturally prevented. These interesting observations by Shah et al. (59) have been made in a pre-clinical model of LPS-induced endotoxemia and need to be confirmed and advanced in a more translational model of sepsis.

1.7 Limitations of Current Mouse Sepsis Models

In recent years, there has been a call in the field for the use of more clinically relevant models in the study of sepsis (61). Whereas the use of LPS allows for easy and highly reproducible inflammatory stimulation, the endotoxemic model has some drawbacks that potentially limit its relevance to the pathophysiology of human sepsis. First, LPS models only examine the effect of endotoxin on the host innate immune system; other interactions between the host and gram-negative bacteria are absent in this context. Second, LPS is usually administered as a bolus; consequently, inflammatory responses are stimulated and resolve acutely. In human sepsis with a bacterial infection, ongoing LPS production as a result of bacterial replication would result in a more chronically inflamed state. Lastly, the sensitivity of humans to LPS is thousands of times higher than in mice, making it difficult to determine how observations in murine endotoxemia models relate to humans (62, 63). Overall, LPS is a useful tool when investigating specific pathways such as TLR signaling, but endotoxemia is not equivalent to a true septic state.

In regard to translational models, CLP can be argued as a more relevant model of sepsis. CLP causes colonization of the peritoneum by multiple species of microbes as well as tissue

necrosis, both characteristics of intra-abdominal sepsis in humans (62). However, it is inherently a more involved technique than LPS administration. Factors including surgeon skill, amount of fluid resuscitation, length of ligation, size and number of punctures, antibiotic dosing regimen, antibiotic type etc. all affect results obtained using this model. Thus, studies using CLP are heterogeneous in their application of the technique, making results harder to interpret and compare (62, 63). It has been argued that CLP's surgical nature and use of gut-derived bacteria are not an accurate representation of the majority of human sepsis experience (64). A more common cause of sepsis in humans is bacterial infection inducing pneumonia, and thus the fundamental basis of the current study was to develop a translational pneumonia model to explore the impact of route of nutrition in sepsis.

2.0 Statement of the Project

The goal of this project was to investigate the effects of the route of nutrition administration in sepsis using a model of sepsis that addresses several of the limitations of previously discussed models. Clinical relevancy was a main point of focus. Mice were not subjected to a nutritional intervention concurrently with the septic insult, but rather 24 hours after infection in a manner analogous to the clinical setting. Bacteria was chosen as the infectious agent, specifically *Klebsiella pneumoniae.* Bacterial inoculum was given via the intratracheal route as opposed to intravenously or intraperitoneally. Primary outcomes were chosen to investigate beyond the acute effects of sepsis and nutrition. From a translational perspective the study was designed to examine longer-term outcomes, most importantly overall survival.

2.1 Aim 1: To determine the effect of nutritional support route on glucose metabolism and how this affects physiology and survival in a bacteremic mouse model of sepsis.

Hypothesis: Infusion of enteral dextrose under bacteremic sepsis conditions preserves euglycemia, normotension, and is associated with lower mortality compared to mice receiving enteral saline or intravenous dextrose.

2.2 Aim 2: To determine the effect of nutritional support route on inflammatory activity, bacterial burden and dissemination, and lung injury.

Hypothesis: Infusion of enteral dextrose under bacteremic sepsis conditions is associated with decreased levels of circulating pro-inflammatory cytokines, lower bacterial burden and dissemination, and less severe lung injury compared to mice receiving enteral saline or intravenous dextrose.

3.0 Materials and Methods

3.1 Animals

All experiments were performed in 10-12week old male C57BL/6J mice purchased from Jackson Laboratory. Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Mice were housed communally in cages provided by the Department of Laboratory Animal Resources (DLAR) prior to the start of experiments but were housed individually once entered into the experimental protocol. Commercial 12in. high round open top plastic containers were used for individual housing during experimentation. These containers were fitted with custom modifications to provide access to food and water. Mice were given *ad libitum* access to autoclave-sterilized water and irradiated lab grade regular chow (LabDiet Prolab RMH 3000 5P76) for the entire duration of housing and experiments. Mice were housed in an IACUC-approved housing facility in the Thomas E. Starzl Biomedical Science Tower under strictly controlled ambient conditions (68-73^{o}F, 30-70% humidity) and a 12hr. light dark cycle (8am-8pm).

3.2 Catherization and Cannulation

Catheters for femoral artery and vein catheterization, and gastric cannulation, were prepared using micro-renathane tubing (MRE025 and MRE050 respectively, Braintree Scientific).

All tubing was gas sterilized with ethylene oxide and stored in sealed sterile packaging until immediately prior to use. The femoral artery and vein were preferred as the site of catheterization (as opposed to the carotid and jugular) based on prior observations that mice recover more quickly after the procedure. In preparation for catheterization, catheters were primed with heparinized saline (20U/mL heparin in 0.9% sterile saline) to minimize blockage due to clotting. Mice were placed in an induction chamber (Tec 3, General Anesthetic Services Inc.) set to 2% isoflurane until unconscious (assessed via toe pinch). Anesthetized mice were then transferred onto a water-warmed surgery platform under a dissection microscope where anesthesia was maintained at 1-2% isoflurane via a face mask. The upper back of the mouse was shaved using a scalpel blade and antibacterial soap, then disinfected using a Kimwipe with 70% ethanol. A 1cm long incision was made between the scapulae before flipping the mouse over into a supine position. The right thigh of the mouse was then shaved using the same method described above. A vertical incision was made where the right thigh meets the lower abdomen, exposing the femoral artery and vein. Surrounding tissue was separated gently using micro-blunted surgical forceps. A blunt-tip 18G needle (Becton-Dickinson) was inserted into the surgical site and tunneled from the femoral incision to the upper back incision. Catheters were threaded through the needle head and the needle head removed. The femoral artery and vein were carefully separated. The femoral artery was tied off at the proximal end with sterile silk sutures (Ethicon) with sufficient constriction to impede blood flow. Another suture was tied further down at the distal end and a tiny incision was made in the femoral artery and the catheter inserted. The catheter was tied firmly in place with suture around the femoral artery, being careful not to constrict the catheter. Finally, the proximal and distal sutures were removed. The femoral vein was next catheterized in the same manner. After catheterization of both vessels, superglue was applied directly to the surgical site to fix the

catheters in position. The skin incision was closed using sterile silk sutures and the surgical site cleaned with iodophore. The mouse was placed in the prone position and catheter patency assessed by withdrawal of blood by syringe and then flushing any visible blood back through the catheter with heparinized saline. A 24in. length of PFA-coated stainless steel wire (A-M Systems) was sutured to the splenius capitis muscles through a scapulae incision and attached to a swivel (more details provided in the inoculation procedure section). Catheters were taped to the wire to provide both structural support and torque to turn the swivel and accommodate the movement of the mice. Lastly, the scapulae incision was sealed with superglue.

For mice instrumented with a gastric cannula, only the femoral arterial catheter was implanted as described above (i.e. no femoral venous catheter). Cannulation took place after suturing closed the femoral surgical site. To begin the cannulation procedure, a section of the left abdomen of the mouse was shaved and sterilized and a 2cm vertical incision made. The fundus of the stomach was visualized, and a sterile 16G needle head was used to create a small hole in the fundus. The gastric cannula was gently inserted through the hole into the lumen of the stomach and secured using prolene sutures (Ethicon). The cannula was routed to the scapulae incision in the same manner as the arterial catheter. Silk sutures were used to close the abdominal surgical site and catheters/cannulae were attached to the metal wire as described above.

Post-surgery, all mice were given a dose of subcutaneous buprenorphine and intramuscular penicillin G at 0.1mg/kg body wt and 0.15mg/kg body wt respectively.

3.3 Inoculum Preparation

Klebsiella pneumoniae (Strain: ATCC 43816) was purchased as a freeze-dried pellet. The pellet was rehydrated in 1mL of tryptic soy broth (TSB) (MP Biomedicals) and diluted in TSB to a total volume of 5mL. Several drops of the solution were pipetted onto a tryptic soy agar (TSA) (MP Biomedicals) plate and spread using a sterile L-spreader. The inoculated plate was incubated in a heated incubator at 37^{o}C overnight. Several colonies were scraped and introduced into 500mL TSB. This solution was incubated overnight in a heated shaker/incubator at 37^{o}C, 300rpm. 10% glycerol was added and the solution aliquoted at 1mL volumes and stored in a –80^{o}C freezer. Inoculation solutions were prepared by aseptically introducing a small portion of the frozen stock described above into 20mL of fresh TSB. This solution was incubated in the heated shaker/incubator overnight at 37^{o}C, 300rpm. 200µl of solution was then transferred into 20mL of TSB and incubated under the same conditions for 2 hours. After 2 hours of incubation, 15mL of solution was separated and spun in a temperature-controlled centrifuge at 3000rpm for 10min. at 4^{o}C. After centrifugation, supernatant was pipetted off and the bacterial pellet was resuspended in cold sterile PBS. The solution was diluted using cold sterile PBS down to an optical density 600nm (OD600) of 0.17. OD600 was measured on 100µl samples in a clear-bottom 96-well plate using a plate reader set to a wavelength of 600nm. A target OD600 of 0.17 was chosen based on prior bacterial growth curves done to correlate OD600 to bacterial concentration in solution. The bacterial solution was then diluted 1:2500 in cold sterile PBS to reach a target concentration of $2x10^5$ colony forming units (CFUs)/mL.

3.4 Inoculation Procedure

After dilution, the inoculum was kept on ice. At the completion of the catheterization procedure, isoflurane levels were raised to 4% for 4min. to ensure sufficient anesthesia for *K. pneumoniae* inoculation. Mice were then suspended by their upper incisors on a thin metal wire as part of a mouse intubation station (Braintree Scientific). A pair of forceps was used to gently pull the mouse tongue away and down from the mouth to block the esophagus of the mouse, ensuring all inoculum entered the lungs through the trachea. $2x10^4$ CFUs in 100µl of PBS was pipetted into the mouth of the mouse at the base of the throat near the vocal cords. Successful inoculation was confirmed by listening for a clicking sound as the mouse breathed, indicating the solution had entered the airways. The tongue was kept under tension for 30s-1min. after inoculation to ensure the complete volume was aspirated. After inoculation, mice were transferred to individual housing units and connected to a pressure transducer and swivel apparatus for monitoring of hemodynamics (details below).

Prior to the start of this project, the validity of the oropharyngeal aspiration technique was confirmed using blue dye. Mice were inoculated with the blue dye and sacrificed immediately after via cervical dislocation. The respiratory and gastric systems were dissected to check for the presence of dye in the lungs and esophagus/stomach. In 6 mice, no dye was visible in the esophagus/stomach whereas the lungs stained blue, consistent with successful aspiration of the dye into the lungs.

3.5 Experimental Setup

The catheters were connected through a swivel to low flow infusion pumps. Catheters were connected to 26G ½in. needles (20G 1in. for gastric cannulae) attached to ~6in. lengths of microrenathane tubing. The non-needle end of the tubing was connected to two-channel quartz lined swivels (Instech Laboratories) designed to rotate 360 degrees, allowing unhindered movement by the mouse. The swivel was suspended using a three-prong extension clamp attached to a ring stand, approximately 15in. above the mouse. 18in. lengths of polyethylene tubing (Braintree Scientific) connected the output of the swivel channels to syringes mounted on a 6-Razel pump. For arterial catheters a pressure transducer (Argon Medical) was connected between the swivel output and the Razel pump to record blood pressure (BP). Syringes on the Razel pumps were pre-filled with 20U heparin in 0.9% saline and set to a flow rate of 12µL/hr. Prior experience working with catheterized mice indicated that this flow rate kept catheters patent and was sufficiently low volume to not impact BP. Pressure transducers were connected to a bioamplifier (CWE Inc.) that interfaced with a DI-720 Data Acquisition System (DATAQ Instruments) connected to a PC WinDaq Data Acquisition Software (DATAQ Instruments) was used to visualize and record BP as a waveform generated in real-time through continuous sampling at a rate of 180 samples/sec.

3.6 Experimental Protocol

Prior to catheterization on Day 1, mice were assigned to intravenous dextrose (IVD), enteral saline (ES), or enteral dextrose (ED) groups. Group sizes were 12, 12, and 14 respectively.

Experiments were conducted in batches of 3, with one IVD, ES, and ED mouse in each batch. Mice receiving IVD were instrumented with a femoral venous catheter and mice receiving ES or ED were instrumented with a gastric cannula. All mice received femoral arterial catheters, through which blood pressure and heart rate were monitored continuously from the start of the experiment. Time 0 was defined as the point of K. pneumoniae inoculation. At 24hr. post-infection (hpi), blood was sampled from catheterized mice through the femoral arterial catheter. BG was measured using a handheld glucometer (Bayer Contour Next). Male C57BL/6J mice from Jackson Laboratories usually have 156-159mg/dL non-fasted baseline BG at 8-16 weeks of age. In this experiment, hyperglycemia and hypoglycemia are terms relative to this value where BG ≥200mg/dL indicates mild hyperglycemia, ≥300 indicates severe hyperglycemia, ≤80mg/dL indicates mild hypoglycemia, and ≤60mg/dL indicates severe hypoglycemia. Blood was spun down in a temperature-controlled centrifuge, 30sec. at 10,000rpm 4^{o}C. Plasma was collected and frozen in liquid nitrogen before transferring to storage in a –80^{o}C freezer. The red blood cell pellet was discarded. After blood sampling was completed, mice were started on a continuous infusion of saline or 50% dextrose in water at a rate of 100µl/hr (equivalent to 40% mouse daily caloric needs) through either the venous catheter or the gastric cannula. At 48hpi, blood was sampled, and BG measured. If BP dropped below a threshold of 61mmHg for more than 1hr. the mouse was removed from the study and terminal procedures performed. If mice survived until 72hpi, a final blood sample and BG measurement was taken. Mice were then placed in an isoflurane induction chamber set to 5% isoflurane for approximately 3min. Once anesthetized, mice underwent a bronchoalveolar lavage (BAL) and were sacrificed via cervical dislocation. Tissues were collected, and samples processed (details below).

3.7 Bronchoalveolar Lavage

After anesthesia, mice were secured in a supine position on a surgical platform. Isoflurane was maintained through a face mask at 2%. A vertical incision was made from the midsection of the mouse up to the throat. The sternum was cut down the middle to expose the thoracic cavity. After retracting tissue to visualize the trachea, an approximately 2in. length of sterile nylon suture (Ethicon) was passed under trachea and tied to form a loose knot. Another nylon suture was used to tightly tie off the left bronchus, ensuring all lavage fluid would enter only the right lung. A 20G safety IV catheter (Smith Medical) was inserted into the trachea and the knot on the trachea tightened to secure the catheter in place. The stylet hub was then carefully removed, leaving only the soft polyurethane catheter inserted. A 1mL syringe filled with 0.6mL sterile PBS with 0.6mM EDTA was attached to the catheter head and the fluid was slowly pushed into the right lung. The attached syringe was then used to draw fluid out of the right lung and deposited into a 1.5mL tube. This process was repeated 3 more times, using 0.5mL of PBS+EDTA each time, for a total volume of 2.1mL. The volume of return was recorded (approximately 1.7mL). 10% buffered formalin was then administered into the right lung at 15cmH_2O to fix the tissue for histology. The right lung was removed after formalin treatment, placed in a 15mL tube containing 5mL of 10% buffered formalin, and stored at 4^{o}C overnight. 250µL of BAL fluid was aliquoted for DiffQuik staining while the rest was spun down at 2000rpm for 5min. at 4^{o}C. After centrifugation, BAL supernatant was separated and snap frozen in liquid nitrogen before long-term storage at –80^{o}C. The remaining cell pellet, if it contained visible red blood cells, was resuspended in red blood cell lysis buffer (Sigma Aldrich) and spun again at 2000rpm for 5min. at 4^{o}C. Supernatant was then discarded, and the cell pellet resuspended in 200mL of sterile PBS + 0.6mM EDTA. A 10µl aliquot of the resuspension was diluted 5-fold in more sterile PBS with 0.6mM EDTA for cell counting.

3.8 Tissue Collection

After sacrifice, the peritoneum of the mouse was cut to reveal the intraperitoneal cavity. Using a sterilized pair of scissors and forceps, the spleen was removed and weighed on sterile weigh paper before transferring into a 5mL tube containing 1mL of sterile PBS. The pancreas was identified and removed and snap frozen in liquid nitrogen. The gallbladder was then carefully removed, taking care not to release its contents on any other tissues, and snap frozen. Several lobes of the liver were removed and snap frozen. Both kidneys were removed and treated in the same manner. The heart was cut at the base, above the atria, removed and snap frozen. Tools were re-sterilized before removing the left lung from the thoracic cavity to avoid contamination affecting bacterial counts. The left lung was placed on sterile weigh paper and cut into two halves. One half was snap frozen while the other was weighed and placed in a 5mL tube containing 1mL cold sterile PBS. The skin on the left leg of the mouse was retracted to reveal both the lower and upper leg muscles. The soleus, gastrocnemius, and vastus were all removed and snap frozen. Subcutaneous fat was sampled from a fat deposit located right under the skin where the left thigh meets the abdomen. Abdominal fat was sampled from gonadal fat deposits in the intraperitoneal cavity. Both fat samples were snap frozen in liquid nitrogen. Tools were sterilized again, and intestines uncoiled to identify the duodenum, proximal and distal jejunum, and ileum. 3mm sections of each region were collected, sterilizing surgical tools between each sample collection. A 3mm section of the colon was also collected. Stool samples from the ileum and colon were collected if available, and snap frozen. All snap frozen samples were stored at –80^{o}C.

3.9 Bacterial Quantification

Left lung and spleen were homogenized in 1mL of cold sterile PBS using a tissue homogenizer. Tissues were homogenized until no visible pieces of tissue remained in the tube (usually 15-30sec.). The homogenizer was sterilized using 100% ethanol and kept cold with ice and dried with a Kimwipe between each sample. Homogenates were then serial diluted down to 10^{-6} in sterile PBS. 10µl of the 10^{-2}, 10^{-4}, and 10^{-6} dilutions were plated out onto TSA, inverted, and incubated at 37ºC overnight. CFUs were then manually counted and the plate that had between 20-200 CFUs was used to calculate bacterial burden as follows:

Bacterial CFUs/mg tissue = (CFU count * Dilution Factor * 100)/Tissue weight in mg

3.10 Right Lung Histology and Lung Injury Scoring

Right lungs in 5mL 10% buffered formalin were stored at 4ºC overnight, then transferred into 70% ethanol. Tissues were embedded in paraffin wax and sent to Histo-Scientific Research Laboratories for hematoxylin and eosin (H&E) staining. A longitudinally cut section from the midline of the right lung, measuring 10µm in thickness, was chosen for staining. Slides were imaged on an Olympus Provis AX700 microscope under bright field settings using a 60x objective. 10 random fields were imaged for each slide. Lung injury was quantified using a modified Murray Scoring system (65), where a score of 1-4 was assigned based on the amount of cellular infiltration, obstruction of airways, and visible space in the lung parenchyma. A higher score on the scale corresponded with worse lung injury. Lung injury scoring was performed by two independent and

blinded researchers. A score for each image was averaged, and the averages for each of the 10 images was averaged again to assign an overall lung injury score to each mouse.

3.11 Differential Quik Stain

150µL of BAL fluid was pipetted into a cytology funnel (ELITechGroup) and loaded into a cytospin machine (ELITechGroup). A glass slide was labeled and loaded directly in front of the cytology funnel. Funnels were spun at 450rpm for 5min. Slides were removed carefully from the cytospin machine and set faceup on a benchtop. A DiffQuik staining kit (Polysciences Inc.) was used to stain the circular cell layer attached to the slide surface. First, 200µl of the methanol formaldehyde fixative solution was pipetted onto each cell layer. After allowing to fix for 1min., slides were oriented vertically, and the fixative was removed by tapping the corner of each slide against an absorbent pad. Slides were placed flat again and 200µl of Eosin Y in phosphate buffer was pipetted onto each cell layer. After 1min., fluid was removed and 200µl of thiazine dye in phosphate buffer was pipetted onto each cell layer. After 30sec., stained slides were loaded into a glass slide holder, rinsed in deionized water for approximately 15sec., and air-dried.

3.12 Differential Blood Count

Differential Quik stained slides were imaged on an Olympus Provis AX700 microscope under bright field settings using a 10x objective. Images were captured using Magnafire software. Multiple fields were imaged for each slide to comprise at least 200 countable cells. Images were

opened on ImageJ (NIH) and counted manually using the Cell Counter plugin. Cell differentiation was based on subjective visual judgment of cell size (in relation to other cells), shape, granularity, and the presence of lobular nuclei. Cells were sorted into 4 categories: macrophages, neutrophils, lymphocytes, and other. Criteria used to determine categorization were as follows:

- Macrophage: slightly larger to much larger than other cells, irregular shape, no granularity, single nucleus
- Neutrophil: smaller than macrophages, imperfectly round, little to no granularity, lobular nucleus
- Lymphocyte: smaller than macrophages, round, no granularity, single round nucleus encompassing the majority of the surface area of the cell
- Other: highly granular cells and cells that could not be reliably categorized were labeled as “other”

Cell differentiation count was performed by two independent and blinded researchers and counts averaged to determine the final results.

3.13 BAL Total Cell Count

10µl of the 5-fold diluted BAL cell suspension was pipetted onto a disposable hemocytometer (INCYTO) and visualized under a bright field microscope using a 10x objective. Cells in four 4x4 grids (each 4x4 grid contains a volume of 0.0001mL) were counted manually and the total BAL cell count was calculated as:

Cells/mL = (Sum of 4 Counts/4) * Dilution Factor of 5 * Volume Correction Factor of 10,000

3.14 Quantification of Hemodynamic Data

Windaq files were opened in Windaq Waveform Browser (DATAQ Instruments) and edited to encompass only the duration of the experimental protocol for each mouse. A new file was saved for each mouse and opened in LabChart Reader 8 (ADInstruments). A correction of +41mmHg was applied to each BP waveform; this correction was necessary as the pressure transducers were positioned 21.94in. above the mice, creating a pressure differential between the mouse and the pressure sensor. The corrected BP was used to generate another waveform by applying the conversion (1/3 systolic BP + 2/3 diastolic BP) to convert BP to mean arterial pressure (MAP). An hourly MAP average was calculated for each hour of experiment time. To calculate heart rate (HR), the Rate tool was applied to the BP waveform. A threshold was manually set to guide peak detection without missing actual heart beats or detecting peaks in noise. A new waveform was generated, plotting a rolling average of beats per minute (BPM) on the y-axis. An hourly BPM average was calculated for each hour of experiment time.

3.15 Biochemical Assays

Plasma insulin was measured via a mouse insulin ELISA kit (Crystal Chem Inc.) per manufacturer protocols. BAL supernatant protein concentrations were measured using a Pierce

BCA Protein Assay Kit (Thermo Fisher Scientific). Plasma and BAL supernatant cytokines were measured via a 23-Plex Mouse Cytokine Assay (Bio-Rad Laboratories). Measured cytokines were IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, CXCL1, MCP-1, MIP-1α, MIP-1β, CCL5, TNF-α, IL-4, and IL-12(p70).

3.16 Statistical Analysis

Statistical analyses were performed using Prism 6 (Graphpad). All variance is presented as ± standard error of the mean. Differences between treatment groups were analyzed using a one-way ANOVA with post-hoc comparisons via Tukey's multiple comparisons test. Differences across time and treatment were analyzed via two-way ANOVA with post-hoc comparisons via Tukey's multiple comparisons test. Fisher's exact tests were performed using 2x2 contingency tables through an online tool provided by Graphpad.

4.0 Results

4.1 Early Enteral Dextrose Infusion Prevents Hemodynamic Collapse in a *Klebsiella pneumoniae* Model of Sepsis

During the first 24hpi, before initiation of fluid administration, MAP did not differ between the three groups ((Fig. 1A, 1B, Table 2; p=0.2241). From 24-48hpi, mice receiving IVD infusion became hypotensive and mortality was 100% by 50hpi. The MAP was similar between the ES and ED groups during the 24-48hpi period, although reduced in both groups compared to the first 24hpi. From 48-72hpi, the mice receiving ED infusion had significantly higher MAP compared to mice receiving ES (p=0.0162), despite both groups exhibiting a fall in MAP compared to their Day 2 values. Overall, the decrease in MAP in the ED group over the three-day protocol was small with the mice remaining in the normotensive range at 72hpi.

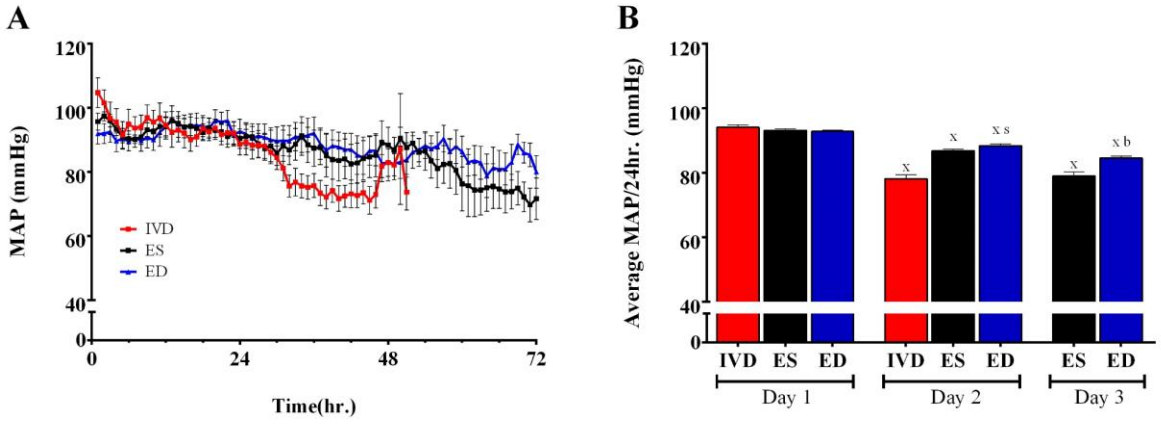

Figure 1. Hourly and Daily Average MAP

A. Hourly MAP average values. B. Daily MAP average values. Hourly MAP averages were calculated as an average of hourly mean values for each surviving mouse at each timepoint. Daily average MAP values calculated based on 24hr. hourly MAP averages. Table 2 lists exact p-values. [x] = compared to previous day's value within same group. [s] = compared to IVD Day 2 value. [b] = compared to ES Day 3 value.

Table 2. Statistical Comparison of Daily Average MAP Values

Treatment	Day	Mean MAP (mmHg)	SEM (mmHg)	Comparisons (p-values)		
IV Dextrose	1	94.1	0.7			
	2	78.2	1.3	$<0.0001^{x}$		
	3	N/A	N/A			
Enteral Saline	1	93.2	0.4			
	2	86.8	0.5	0.0036^{x}	$<0.0001^{s}$	
	3	79.1	1.3	$<0.0001^{x}$		
Enteral Dextrose	1	92.8	0.5			
	2	88.4	0.56	0.0053^{x}	$<0.0001^{s}$	
	3	84.6	0.63	0.047^{x}		0.0162^{b}

Two-way ANOVA was used to compare all groups at all time points with post-hoc testing via Tukey's multiple comparisons test. ^{X} = compared to previous day's value within same group. ^{s} = compared to IVD Day 2 value. ^{b} = compared to ES Day 3 value.

The pattern of HR over time in the three experimental groups was similar to that of MAP, despite the IVD group unexpectedly having a lower HR in the 0-24hpi period compared to the ES and ED groups (Fig. 2A, 2B, Table 3). Mice receiving ED infusion maintained a higher HR over the course of the three-day experiment compared to mice receiving IVD or ES infusion (ED vs. ES on day 3, $p<0.0001$). Mice receiving IVD became markedly bradycardic during the 24-48hpi period, accounting for the hypotension that manifested prior to death.

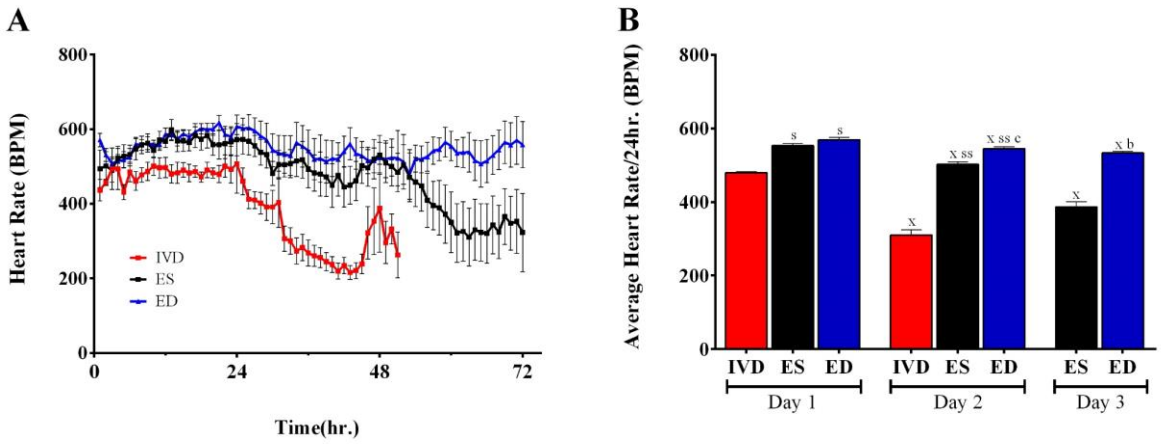

Figure 2. Hourly and Daily Average HR

A. Hourly HR average values. B. Daily HR average values. Hourly HR averages were calculated as an average of hourly mean values for each surviving mouse at each timepoint. Daily average HR values calculated based on 24hr. hourly HR averages. Table 3 lists exact p-values. ^{x} = compared to previous day's value within same group. ^{s} = compared to IVD Day 1 value. ^{ss} = compared to IVD Day 2 value. ^{c} = compared to ES Day 2 value. ^{b} = compared to ES Day 3 value.

Table 3. Statistical Comparison of Daily Average HR Values

Treatment	Day	Mean HR (BPM)	SEM (BPM)	Comparisons (p-values)		
IV Dextrose	1	479	4			
	2	310	15	$<0.0001^{x}$		
	3	N/A	N/A			
Enteral Saline	1	553	6	$<0.0001^{s}$		
	2	502	7	0.0005^{x}	$<0.0001^{ss}$	
	3	387	14	$<0.0001^{x}$		
Enteral Dextrose	1	569	6	$<0.0001^{s}$		
	2	544	6	0.317^{x}	$<0.0001^{ss}$	0.0039^{c}
	3	531	5	0.885^{x}		$<0.0001^{b}$

Daily average HR values calculated based on 24hr. hourly HR averages. Two-way ANOVA was used to compare all groups at all time points with post-hoc testing via by Tukey's multiple comparisons test. ^{X} = compared to previous day's value within same group. ^{s} = compared to IVD Day 1 value. ^{ss} = compared to IVD Day 2 value. ^{c} = compared to ES Day 2 value. ^{b} = compared to ES Day 3 value.

4.2 Early Enteral Dextrose Infusion Preserves Euglycemia and Insulin Sensitivity in a *Klebsiella pneumoniae* Model of Sepsis

At 24hpi, mice in each of the three groups had similar BG levels, all at the lower end of the normoglycemic range (Fig. 3A). Mice receiving IVD infusion became severely hyperglycemic by 48hpi, while mice receiving ES infusion gradually progressed to hypoglycemia from 24-72hpi. In contrast, mice in the ED group exhibited a mild hyperglycemia at 48hpi that was maintained through to the end of the protocol at 72hpi. Plasma insulin levels were comparable, and in the normal range, for all three groups at 24hpi (Fig. 3B). In the ES group, plasma insulin remained low through 72hpi. In contrast to the BG response, the ED and IVD groups exhibited large and comparable increases in plasma insulin at 48hpi (IVD 7.5 ± 1.6 and ED 5.9 ± 2.7pg/mL; p=0.14 respectively). The combination of high BG and high plasma insulin in the IVD group at 48hpi is consistent with a state of decreased insulin sensitivity compared to the ED group that exhibited much lower BG at comparable plasma insulin levels.

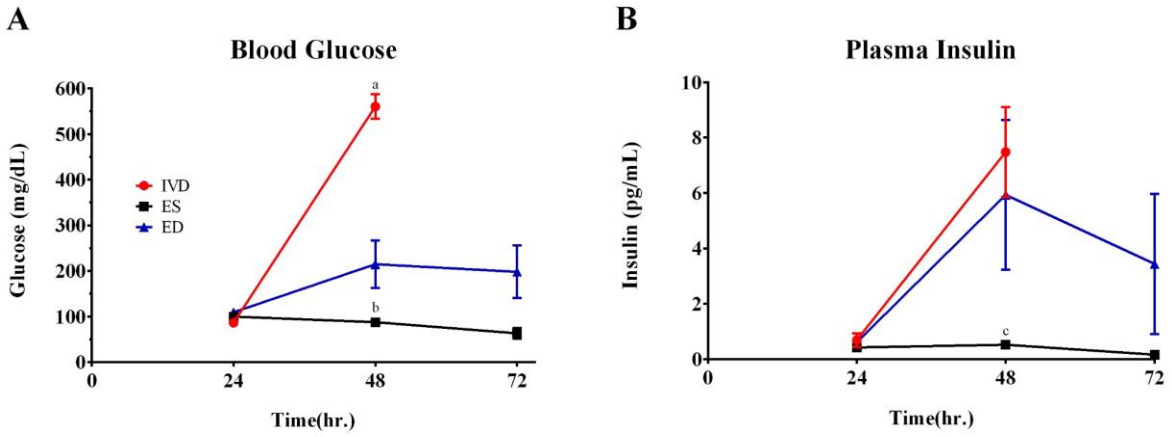

Figure 3. Average BG and Plasma Insulin Levels at 24, 48, and 72hpi

A. Average blood glucoses taken at 24hr. intervals post infection. B. Average plasma insulin values measured from plasma samples collected at 24hr. intervals post infection. Two-way ANOVA was used to compare groups at all timepoints with Tukey's multiple comparisons test. [a] = IVD compared to ED at 48hpi, $p<0.0001$ [b] = ES compared to ED at 48hpi, $p=0.0149$ [c] = IVD compared to ES at 48hpi, $p=0.0382$

4.3 Early Enteral Dextrose Can Attenuate Pro-Inflammatory Responses in a *Klebsiella pneumoniae* Model of Sepsis

The responses of six cytokines with relevance to sepsis are presented in Fig. 4 and Appendix A. For the ED and ES groups, the levels of IL-1β, IL-6, TNF-α, G-CSF, MCP-1, and IL-10 were the same at 24hpi and the pattern of response over the course of the experiment from 24-72hpi was comparable. In contrast to the ED group, the IVD group exhibited marked elevations in IL-6, IL-10, MCP-1, and G-CSF at the 48hpi timepoint (Fig. 4C, D, E, and F). For the early response cytokines, IL-1β and TNF-α, plasma levels trended downwards in all three groups across the 24-72hpi time period (Fig. 4A and B).

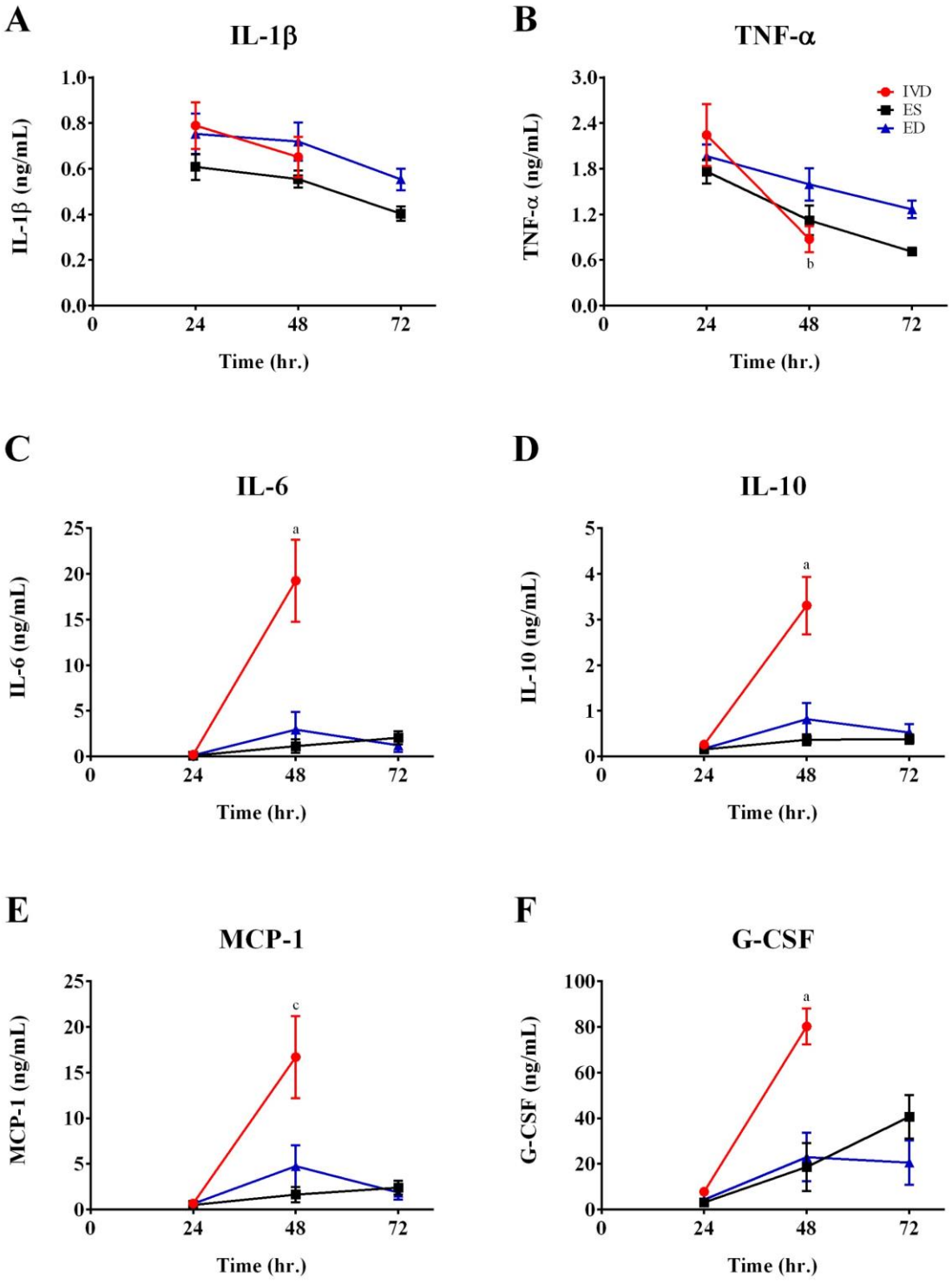

Figure 4. Plasma Cytokine Concentrations at 24, 48, and 72hpi

A. IL-1β B. TNF-α C. Il-6 D. IL-10 E. MCP-1 F. G-CSF. Two-way ANOVA with Tukey's multiple comparison tests was used to compare groups at all timepoints. [a] = IVD compared to ES or ED at 48hpi, $p<0.0001$ [b] = IVD levels compared at 24 and 48hpi, $p=0.0024$, [c] = IVD compared to ES or ED at 48hpi, $p=0.0002$ and $p=0.0015$ respectively.

4.4 The Route of Nutrition Administration does not Affect Bacterial Burden or Dissemination in a *Klebsiella pneumoniae* Model of Sepsis

No differences were seen in bacterial burden of the lungs between any of the three groups at time of sacrifice (Fig. 5A). Similarly, no differences were observed in splenic CFUs between the three groups, consistent with a comparable degree of bacterial dissemination from the lungs under each of the three experimental conditions (Fig. 5B).

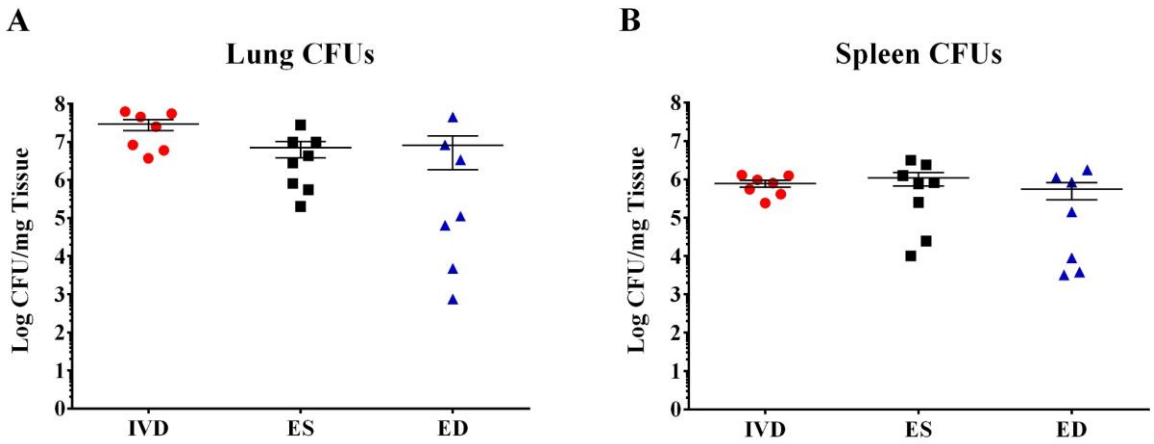

Figure 5. Bacterial Growth in Left Lung and Splenic Tissue

A. CFUs grown from tissue homogenate of the left lung, plated on TSA. B. CFUs grown from splenic tissue homogenate, plated on TSA. No differences were observed between any groups, tested by One-way ANOVA with Tukey's multiple comparisons test.

4.5 Lung Injury and Inflammation May Be Exacerbated by IV Administration of Dextrose in a *Klebsiella pneumoniae* Model of Sepsis

There was no difference between the ED and ES groups in BAL total protein, BAL cell count, BAL neutrophils, or histological lung injury (Fig. 6A-D). For the IVD group there was a significant elevation in BAL total protein compared to the ED group, accompanied by strong trends

for increases in BAL cell count and BAL neutrophil proportion (Fig. 6C and D; p=0.0929 and p=0.1079 respectively).

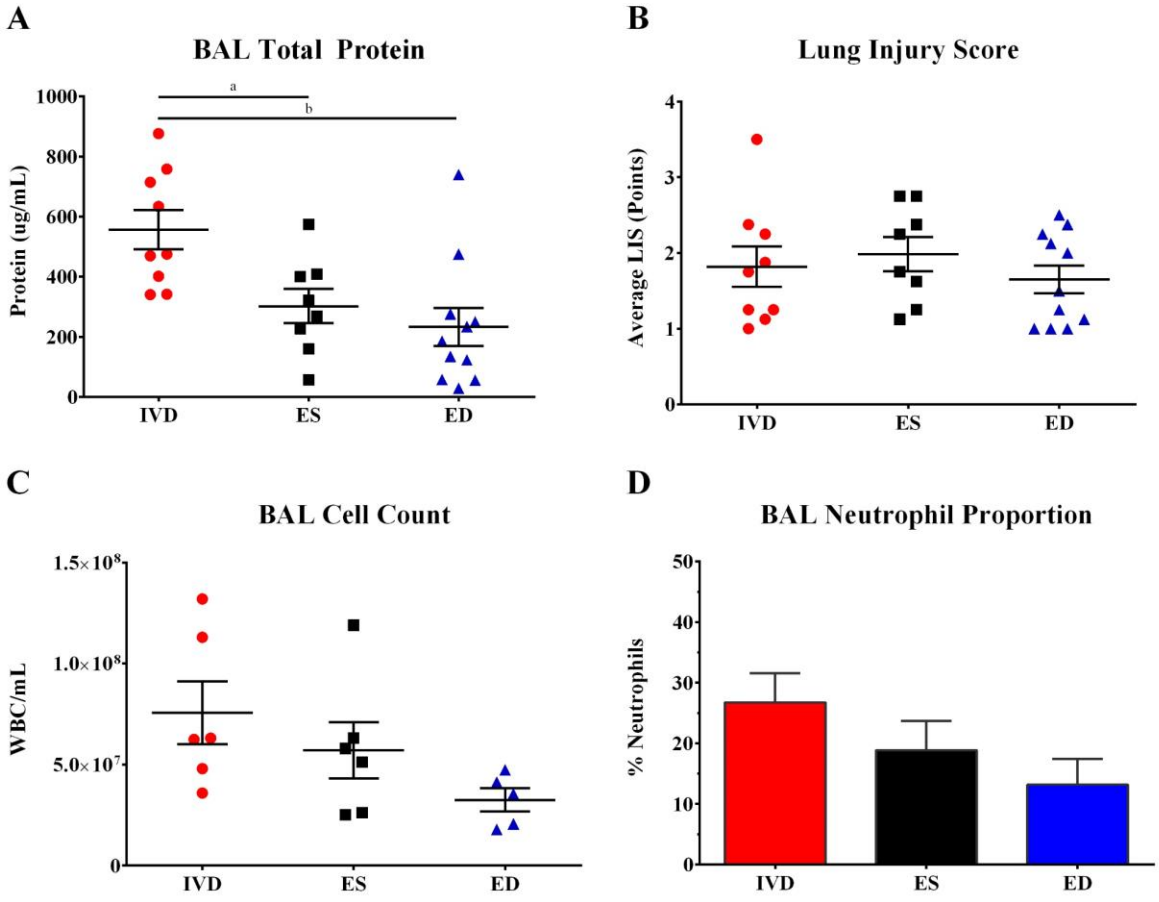

Figure 6. Markers of Lung Injury and Inflammation

A. BAL protein concentration measured from the BAL supernatant. [a] = IVD compared to ES group, p=0.0307 [b] = IVD compared to ED group, p=0.0027. B. Histological assessment of H&E stained sections of the right lung using a 1-4 point scale. C. White blood cell concentrations in BAL supernatant. D. Within white blood cells in the BAL supernatant, the proportion of WBCs identified as neutrophils (IVD n=8, ES n=8, ED n=11). One-way ANOVA was used for all 4 outcomes, with Tukey's multiple comparisons test.

4.6 Early Enteral Dextrose Infusion Improves Survival in a *Klebsiella Pneumoniae* Model of Sepsis

Despite a lack of difference in bacterial burden and lung injury, mice receiving ED infusion had a significantly lower mortality compared to mice receiving ES at 72hpi (Fig. 7; ED = 27% vs ES = 78%; p=0.0002). The IVD group had the highest rate of mortality reaching 100% by 50hpi. In the two groups receiving dextrose infusion (IVD and ED), hyperglycemia was significantly associated with increased mortality. Of the combined 22 mice in the two dextrose infusion groups, 15 (all 11 mice receiving IVD and 4 mice receiving ED) progressed to hyperglycemia and 7 maintained euglycemia. Among the euglycemic mice there were no deaths at 72hpi, whereas 14 out of 15 of the hyperglycemic mice were dead at 72hpi ($p<0.0001$ using Fisher's exact test). In mice receiving ES, hypoglycemia was also significantly associated with increased mortality. Out of 9 mice infused with ES, 7 progressed to hypoglycemia and 2 remained euglycemic. Both euglycemic mice remained alive at 72hpi, whereas all 7 hypoglycemic mice died before the end of the experiment (p=0.0278 using Fisher's exact test).

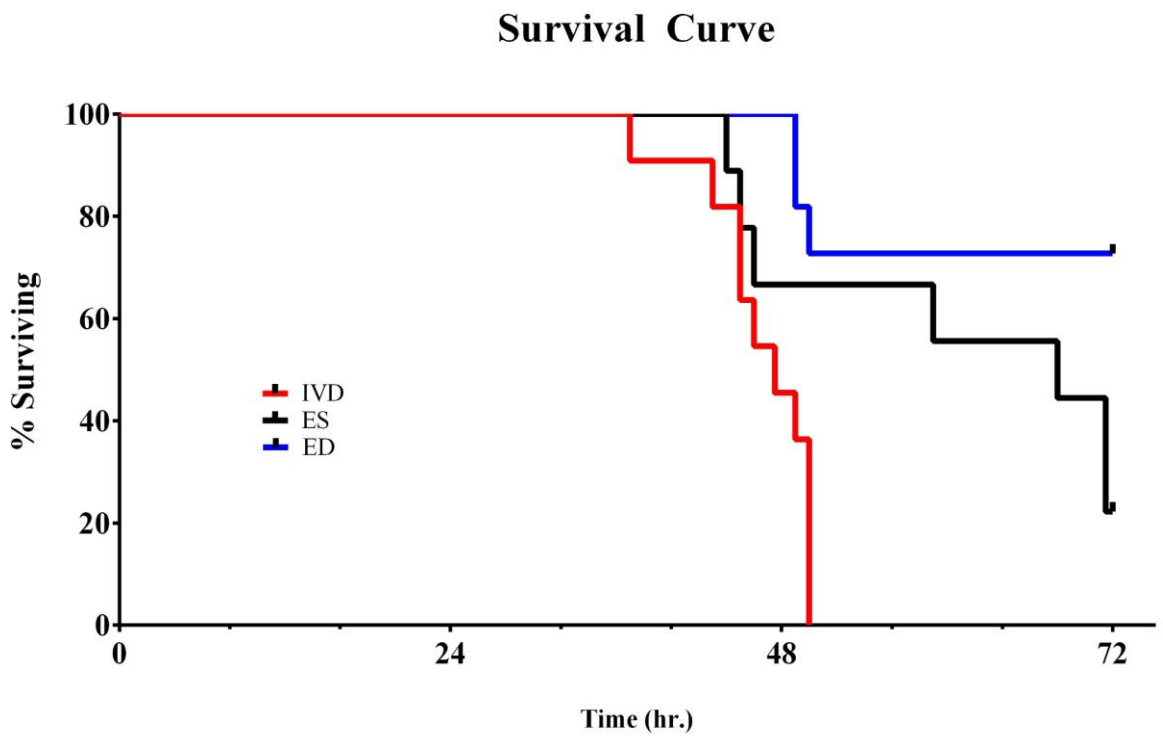

Figure 7. Kaplan-Meier Survival Curve

Survival curve showing mortality in mice receiving IVD, ES, and ED. Survival curves were significantly different based on Mantel-Cox log-rank test (p=0.0002). Mice that fell below a BP threshold of 61mmHg for at least an hour were sacrificed; time of sacrifice was used as the time of death in the survival curve. This threshold was chosen based on experience working with septic models in which it was commonly observed that mice exhibiting a BP <61mmHg did not survive longer than ~3hr.

5.0 Discussion

Sepsis as a public health and global healthcare issue has seen increased research focus in recent years. In 2015, definitions that had remained effectively unchanged for two decades were finally updated (8, 66, 67). Similarly, there has been a re-evaluation of priorities in how research in sepsis needs to be conducted. In 2019, the National Institute of General Medical Sciences (NIGMS) published a notice of information regarding its priorities in sepsis research, emphasizing the need for increased clinical research funding. In addition, NIGMS stressed a need for more translationally relevant mechanistic studies looking at the pathophysiology of sepsis at the genetic, molecular, cellular, and tissue level to inform the discovery of new sepsis biomarkers/pathways and development of more refined diagnostic methods (68). In the pre-clinical sphere of sepsis research, much attention has been directed at the weaknesses of previously established animal models of sepsis and their potential limited clinical relevance. Issues raised include the interventions to induce sepsis, homogeneity of the animals used, focus on only male animals, and the use of experimental timeframes that do not reflect the time course of sepsis, among many other concerns (61). The aim of the current study was to address many of these limitations using a *Klebsiella pneumoniae* model to answer the highly relevant clinical question of whether an enteral route of administration of dextrose can improve cardiovascular homeostasis, metabolism, inflammation, lung injury and survival compared to a parenteral route of dextrose administration.

5.1 Why Use a *Klebsiella pneumoniae* Model of Sepsis?

Pneumonia is the leading cause of sepsis, accounting for around half of all documented cases (based on data from developed countries) (69). Infectious diseases, especially lower respiratory tract infections (LTRI), disproportionately affect low-income countries (70); thus, it is highly likely that pneumonia-induced sepsis occurs at an even higher incidence than the literature reports. Of pneumonia causing bacteria, *K. pneumoniae* was the more clinically relevant choice compared to, for example *S. aureus* or *S. pneumoniae*; Gram-negative bacteria are more commonly found in septic ICU patients than Gram-positive bacteria (71). Intratracheal inoculation of bacteria, rather than intravenous or intraperitoneal, was used to more realistically model LTRI. Titration of the dosing to achieve ~20% mortality at 72hpi was chosen to reflect the timing of mortality due to severe sepsis seen in the ICU. Penicillin G administration and a 24hr. delay on dextrose/saline infusion were included in this model to more accurately simulate the timing and treatment of sepsis in patients. B6 mice were chosen for their T_h1 -skewed immune response (72). As B6 mice are particularly robust in clearing intracellular bacterial infections like *K. pneumoniae*, any significant mortality and morbidity benefits observed would be more credible than if seen in other conventional strains, such as Balb/c. Although the *Klebsiella pneumoniae* model does not address every issue surrounding animal sepsis models, there are multiple advantages compared to many other pre-clinical murine models.

5.2 Metabolic Benefits of ED

In the current study, outcomes indicating severity of bacterial burden, bacterial dissemination, and lung inflammation and injury did not show significant differences overall between the three groups. Despite comparable degrees of infection and lung injury, a significant survival benefit was observed with provision of ED. One possible explanation for the survival benefits of ED is the maintenance of metabolic control. Both hyperglycemia and hypoglycemia were significantly associated with mortality by 72hpi in this study, with all 21 deaths being accompanied by one or the other state of dysglycemia. Among the 10 survivors of the ED and IVD groups, 9 remained euglycemic for the duration of the experiment. Although the average BG in mice receiving ED indicated mild hyperglycemia at 48hpi, the distribution of BG values was bimodal and the average skewed by the presence of a few non-surviving mice that became severely hyperglycemic. Indeed, all surviving mice receiving ED remained euglycemic for the duration of the experiment. Despite widely disparate BG levels between the ED and IVD groups at 48hpi, both interventions elicited a similar and marked insulin response. Combining the BG and insulin data, the data are consistent with ED preserving insulin sensitivity to prevent hyperglycemia, while simultaneously mitigating the risk of hypoglycemia. It remains to be determined whether maintaining metabolic homeostasis accounts for the survival benefits of ED in our model, and what underlying mechanisms account for preservation of insulin sensitivity and insulin secretion.

5.3 Mechanistic Pathways Impacting Glucose and Insulin Regulation in Sepsis

Activation of pro-inflammatory and inflammatory pathways can negatively impact insulin sensitivity (73, 74) and could account for the metabolic and mortality responses we observed with the IVD intervention. In humans, circulating cytokine responses during sepsis have been studied extensively as a marker of disease severity, evolution of organ dysfunction, and predictor of mortality; Il-1β, TNF-α, IL-6, IL-10, MCP-1, and G-CSF were chosen as cytokines of interest in the current study due to their role in the generation of the inflammatory response and relevancy in current sepsis research (9, 10, 75). Based on what is known about the complex pattern of cytokine responses to sepsis (76) and the limited timepoints available in our study, it is likely that IL-1β and TNF-α peaked in the first 24hpi period and subsequently were in decline from 24-72hpi. In contrast, for the IVD group in particular, IL-6, IL-10, MCP-1, and G-CSF, were highest at 48hpi. Although our data cannot unequivocally determine when specific cytokine peaks occurred, the data indicate that induction of IL-1β and TNF-α precedes the other 4 cytokines. This temporal profile suggests a pro-inflammatory response generated through NLRP3 inflammasome activity, an innate immune system pathway activated by TLR4-LPS binding, and subsequent NF-κB pathway signaling (11, 76). Stimulation of the inflammasome pathway, referred to as the priming phase, causes upregulated transcription of pro-IL-1β, pro-IL-18, NLRP3, and pro-caspase-1, with a second activating signal (PAMP/DAMP recognition, ROS, extracellular ATP etc.) inducing the formation of the NLRP3 inflammasome complex. This complex cleaves pro-caspase-1 into active caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 (76). Given that the NLRP3 inflammasome is an innate immune system component, its activation occurs early in the immune response. Indeed, IL-1β and IL-18 are among the first pro-inflammatory cytokines released by macrophages and dendritic cells in response to pathogen recognition (76). TLR4-LPS

induced NF-κB signaling also triggers the expression of TNF-α, another early pro-inflammatory cytokine (12). TNF-α has been implicated as a mediator of inflammasome activation, possibly through its stimulatory effect on ROS generation, as well as its ability to provide both a priming signal by activating the NF-κB pathway and an activating signal to induce inflammasome complex formation (77). Although LPS acts as the initial stimulus to trigger TNF-α expression and NLRP3 inflammasome activity, both TNF-α and IL-1β are subsequently capable of priming the NF-κB pathway through binding of membrane-bound TNFR and IL-1R1 respectively, and thus are able to propagate their own and each other's expression after initial PAMP recognition (11). Based on this relationship, it could be assumed that TNF-α and IL-1β are expressed in concert and would be consistent with our observations in the *K. pneumoniae* model. Unfortunately, the 23-plex cytokine assay did not measure IL-18; if the IL-18 temporal profile matched those of IL-1β and TNF-α, it would reinforce the concept of NLRP3 inflammasome involvement.

The pro-inflammatory cytokine IL-6 has been a focus in sepsis research, serving as both an indicator of sepsis severity in clinical trials, and as a predictor of mortality (78). IL-1β, TNF-α, and IL-6 are often considered collectively and cross-sectionally when discussing inflammation, but there is evidence that IL-6 activation pathways are regulated and/or initiated by IL-1β and TNF-α (79-81). Thus, peak IL-6 expression should occur later relative to the other two pro-inflammatory cytokines, as is seen in our model. The observation that provision of ED attenuates inflammation as indicated by significantly lowers IL-6 levels compared to IVD suggests that this occurs independent of upstream NRLRP3 inflammasome activity, although it is possible that IL-1β and TNF-α peaked at higher levels in the IVD group compared to the ED group in the first 24hpi. Whatever the mechanism, it is clear that ED attenuates the inflammatory response to sepsis compared to IVD and potentially could account for the more favorable metabolic environment.

Another differentiation between IVD and ED is that delivery of dextrose through the enteral route will preferentially stimulate secretion of gut hormones.

Incretins are insulin-stimulating hormones released by enteroendocrine cells in the proximal and distal portions of the small intestine in response to a nutrition. These hormones enter the circulation and stimulate release of insulin from pancreatic islets in a blood-glucose dependent manner (60). Although predominantly considered an insulin secretagogue acting on pancreatic β-cells, incretin receptors are also found in heart and brain tissue and can affect cardiac output and appetite (60). Relevant to the current study, incretins possess inflammation-attenuating effects. Incretins can inhibit IL-1β production *in vitro,* and decrease circulating pro-inflammatory cytokine levels (IL-1α, IL-1β, IL-6, TNF-α, IFNg) *in vivo* (82, 83). An earlier study from our laboratory showed circulating incretins were increased by ED and played a major role in preserving metabolic homeostasis and attenuating inflammation in an LPS model of murine endotoxemia (59). Thus, it is likely that the metabolic, inflammatory, and survival benefits of ED over IVD in our *K. pneumoniae* model, are at least in part, attributable to the actions of incretins. Related to metabolism, the current data demonstrate hyperglycemia in mice receiving IVD, hypoglycemia in mice receiving ES, and preserved insulin sensitivity and euglycemia in mice receiving ED. As incretins have been shown to possess insulin sensitivity-preserving abilities (84) and stimulate insulin secretion without risk of hypoglycemia, the favorable glycemic outcome in the ED group is potentially incretin mediated, although direct interventional studies would be needed to directly confirm the mechanism.

5.4 Translational Relevance

Currently, there is no standard practice for implementing caloric provision in sepsis. However, it is widely accepted that caloric support works directly to prevent/treat ICU-AW and PICS (26, 27, 85), sepsis-related conditions that carry severe long-term mortality and morbidity-related consequences. Therefore, patients must be fed. The results of the current study suggest that, with respect to route of nutrition, provision of dextrose through the enteral route is significantly less risky than through the intravenous route. Hemodynamically, mice receiving ED were able to preserve cardiovascular homeostasis compared to mice receiving IVD, with the latter becoming severely hypotensive and brachycardic; likely due to a stronger pathogenic inflammatory response in mice receiving IVD as indicated by elevated circulating IL-6 levels. Regarding survival, the 100% mortality rate of mice receiving IVD compared to 78% in mice receiving ES indicates that provision of IVD may actually be harmful during the acute phase of sepsis, while only 27% mortality in mice receiving ED suggests enteral nutrition may be beneficial. BG responses mirror the mortality data. Despite equal dextrose administration, mice receiving ED experienced less hyperglycemic events compared to mice receiving IVD, all of which became hyperglycemic. Moreover, all of the ES mice became hypoglycemic in contrast to none of the ED mice. Both hyperglycemia and hypoglycemia are detrimental in septic patients and ideally, both need to be prevented (33, 48). Our results suggest that with regard to dextrose administration the enteral route is optimal.

5.5 Limitations and Future Directions

Given that caloric provision is an important component of sepsis care, the evidence presented in the current study supports the enteral route as the preferred method to deliver nutrition. However, there are some limitations in the experimental design of this study that warrant discussion, and gaps in knowledge exist that invite further research. In the current study, 10 week-old male B6 mice were used; as sepsis disproportionately affects elderly humans, the use of 10 week-old mice (analogous to 20 yr. old humans) limits this model's translational relevance to human sepsis. No female mice were used in this study; thus, any gender specific differences on route of nutrition were left unexplored. Collection of samples from terminal procedures occurred at different times depending on the survival of the mice, making systematic comparisons between groups less rigorous. Dextrose was infused at a constant rate equating to 40% daily caloric needs; the effects of low-level vs. full feeding, a question of significant clinical relevance, were not specifically investigated. Mice were given free access to food, and it has been observed in prior work that severity of sepsis/endotoxemia is inversely associated with the amount of food eaten. Mice receiving ED infusion ate more food, on average, compared to mice receiving IVD and ES (data not shown), creating an additional variable between groups. Nevertheless, the data from the current study provide novel insights into the potential benefits of enteral nutrition that would be much harder to establish in the very heterogeneous populations of septic patients in the ICU environment.

Results in our catheterized *K. pneumoniae* model indicate early enteral dextrose administration confers beneficial effects in the setting of sepsis, specifically in glucose regulation, attenuation of inflammation promoting cardiovascular homeostasis, and overall survival. Contrastingly, intravenous dextrose may precipitate dysregulation of glucose metabolism, an

excessive inflammatory response leading to severe hypotension and brachycardia, and increased mortality. Overall, our data create a focus for further study on the role of incretins in nutritional support in sepsis. Using this catheterized model, circulating incretin levels could be measured, and exogenous incretin administration and pharmacologic blockade interventions could be used to directly establish mechanism. In humans, a retrospective analysis of a cohort of patients directly comparing IVN vs. EN could provide more credible evidence towards possible EN benefits than previous observational studies, although these analyses are made difficult by the heterogeneity in patient populations, lack of standardization of nutritional support amounts, timing, and formulations, and subjectivity in diagnosing sepsis. Ultimately randomized prospective interventional human trials comparing intravenous versus enteral nutrition or administration of exogenous incretin hormones will be necessary to impact clinical practice in the field of sepsis.

Appendix: Plasma Cytokine Measurements

Table 4. Plasma Cytokine Levels at 24, 48, and 72hpi

Treatment	Time (hr.)	IL-1β (ng/mL)	TNF-α (ng/mL)	IL-6 (ng/mL)	IL-10 (ng/mL)	MCP-1 (ng/mL)	G-CSF (ng/mL)
IV Dextrose	24	0.79 ± 0.10	2.24 ± 0.41	0.20 ± 0.01	0.26 ± 0.03	0.65 ± 0.11	7.76 ± 1.34
	48	0.65 ± 0.09	0.88 ± 0.17	19.25 ± 4.51	3.31 ± 0.63	16.70 ± 4.52	80.22 ± 7.94
	72	n/a	n/a	n/a	n/a	n/a	n/a
Enteral Saline	24	0.61 ± 0.06	1.76 ± 0.16	0.09 ± 0.02	0.15 ± 0.02	0.49 ± 0.06	3.07 ± 0.93
	48	0.55 ± 0.04	1.12 ± 0.19	1.12 ± 0.74	0.37 ± 0.12	1.64 ± 0.83	18.60 ± 10.51
	72	0.4 ± 0.03	0.71 ± 0.04	2.04 ± 0.73	0.38 ± 0.11	2.39 ± 0.78	40.64 ± 9.54
Enteral Dextrose	24	0.75 ± 0.09	1.97 ± 0.15	0.12 ± 0.03	0.17 ± 0.02	0.62 ± 0.14	4.29 ± 1.29
	48	0.72 ± 0.08	1.60 ± 0.21	2.93 ± 1.96	0.82 ± 0.36	4.74 ± 2.27	22.92 ± 10.62
	72	0.55 ± 0.05	1.27 ± 0.11	1.21 ± 0.71	0.52 ± 0.19	1.86 ± 0.80	20.55 ± 9.72

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