Modified Clinical Monitoring Assessment Criteria for Multi-Organ Failure during Bacteremia and Sepsis Progression in a Pig Model

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Abstract

Objective: Sepsis animal models commonly fail to predict clinical efficacy of novel therapeutics in humans because most animals are more resistant to pathogens and sepsis is not measured or evaluated in the same manner. We modified the third international consensus clinical criteria for sepsis and septic shock (Sepsis-3) to evaluate and diagnose two swine models of sepsis, as pigs are similar to humans in their immunology and susceptibility to pathogens.

Materials and Methods: Eighteen female Yorkshire Swine, anesthetized or conscious, received intravenous E. coli (clinical isolate) infusions of 3 x 10^8 CFU/kg over 4 hours or 2 x 10^9 CFU/kg over 9.5 hours, respectively. Symptoms, vitals and blood were monitored throughout for disease progression.

Results: We established a swine-specific Sepsis-3 (ss-Sepsis-3) system, including clinically relevant Sequential (sepsis-related) Organ Failure Assessment (SOFA) scoring criteria for pigs, by adapting human clinical protocols. Both models resulted in acute symptoms of sepsis (SOFA ≥ 2); however, septic shock developed in only 3 of 6 anesthetized animals (SOFA ≥ 2, high lactate levels and requirement for vasopressors). Additionally, acute kidney injury was observed in 2 of 6 conscious animals, defined using a modified clinical Risk, Injury, Failure, Loss of kidney function and End-stage kidney disease (RIFLE) system. Anesthetized infected animals also displayed decreased mean arterial pressures and lower temperatures compared to conscious animals, likely caused by anesthesia. Cytokine profiles were complex and varied in both models.

Conclusions: The ss-Sepsis-3 scoring system described here quantifies the effects of pathogen infusion and disease severity in real-time. Using the same clinical pathogen isolate, the severity of disease progression in the anesthetized model was increased due to the confounding effects of anesthesia. This scoring system provides a framework for adaptation to other large animal models of sepsis to evaluate disease progression, mechanism and effectiveness of therapeutic drugs or devices.

Keywords: Sepsis; Sepsis-3; SOFA; Blood culture; Organ dysfunction; Acute kidney injury; Pig model

Introduction

Many potential sepsis treatments have been evaluated in clinical trials over the past thirty years; however, standard practice remains limited to antibiotics and fluid therapy. A major cause of this failure to develop new therapies is that existing animal models are poor predictors of human clinical outcome in sepsis. Most animal models of sepsis are conducted in species that have increased resistance to pathogens compared to humans, for example, rodents and baboons [1]. Given that FDA guidelines suggest that all new drug applications include data from two species, one of which must be non-rodent [2], there is significant unmet need for large animal models of sepsis that more closely represent human sensitivities to infection. The pig’s immune system has over 80% similarity to humans, compared to <10% for mouse [3]; their coagulation system is similar to humans [4]; and their size and blood volume enables real-time monitoring and temporal clinical evaluation of sepsis progression.

Pre-clinical sepsis model disease symptoms and outcomes are rarely measured or evaluated in an equivalent manner to clinical practice, and specific organ dysfunction measurements for all organ systems are beyond the capabilities of most research facilities. In 2016, the third international consensus clinical criteria for sepsis and septic shock (Sepsis-3) were published [5], providing updated clinical evaluation criteria for sepsis and a more clinically relevant staging system compared to the 2001 guidelines [6,7]. Sepsis-3 classifies sepsis as a disease involving systemic infection that leads to organ dysfunction, as defined using Sequential (sepsis-related) Organ Failure Assessment (SOFA) scoring criteria, which incorporate measures of cardiac, renal, hepatic, respiratory, neurologic and coagulation function [5]. Septic shock is defined by a SOFA ≥ 2 and the need for vasopressors with a lactate of > 2mM [5]. Evaluating animal models of sepsis using this type of SOFA-like scoring system could have multiple benefits by: 1) providing an alternative endpoint to mortality, improving animal welfare and reproducibility, 2) allowing more direct comparisons of outcomes within animal studies with respect to clinical outcomes observed in human patients, and 3) generating information relevant for translation of pre-clinical results to clinical trials.

The majority of large animal models of sepsis are conducted under anesthesia using intravenous or intraperitoneal pathogen inoculations with a single type of pathogen. However, certain aspects of pre-clinical model design (e.g., surgical procedure, type or route of anesthesia) can alter symptoms, change susceptibility to infection, or interfere with measurements. For example, general anesthesia itself can result in hypothermia [8,9], which can mask fever or further exacerbate the hypothermia commonly associated with the development of sepsis. General anesthetics can also produce cardiodepressive effects by decreasing systemic blood pressure due to reduced sensitivity of the baroreflex, peripheral vasodilation and decreased systemic vascular resistance [10,11]. Additionally, both general anesthesia and surgical stress have been implicated to increase susceptibility to endotoxin [12,13].

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Based on the human Sepsis-3 system, we generated a modified swine-specific Sepsis-3 (ss-Sepsis-3) scoring system and swine-specific SOFA (ss-SOFA) scoring criteria. We used these criteria to evaluate sepsis in a pilot study of anesthetized versus conscious swine models of sepsis produced using the same clinical isolate of *Escherichia coli* (*E. coli*) [14]. This approach allowed us to determine the potential usefulness of the scoring system as applied to these swine models, and to better understand the effects of anesthesia in relation to sepsis-related disease progression and organ dysfunction.

### Methods

#### Study details

Approval for the study was obtained from the Institution Animal Care and Use Committee (protocol number 14-03-2519) and the Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Materiel Command (USAMRMC) Office of the Department of Defense (DOD). The work was conducted at an AAALAC accredited and USDA registered facility and in accordance with NIH guidelines. Outbred juvenile Yorkshire swine (35-50kg) were given intravenous *E. coli* (clinical isolate 41949 obtained from Brigham and Women’s Hospital, Crimson Biorepository, USA) [14]. In total, 18 animals were used in this study; 12 were given *E. coli* in 2 groups (n=6 per group) while the animals were anesthetized or conscious. A total of 6 animals received identical procedures without infusion of *E. coli* (n=4 anesthetized and n=2 conscious).

Cannulas were placed surgically in the jugular vein and carotid artery for pathogen infusion and clinical monitoring. Animal vitals were monitored, and blood samples were taken for the following analysis: blood culture, complete blood count, blood gas, blood chemistry, clotting profile, enzyme linked lectin-sorbent assay, c-reactive protein and cytokines. Organ samples were obtained following the procedure. Full details are described in the Supplementary Information 1.

#### Results

The human Sepsis-3 and SOFA scoring system, which evaluates 6 major organ systems: respiratory, coagulation, cardiovascular, hepatic, central nervous system (CNS) and renal, were modified to reflect differences between human and swine, with five of the six organ systems taken into consideration for this model (Table 1 and detailed in Supplementary Information 2). Experimental ss-SOFA scores, measured over time (Figure 1A), showed anesthetized animals exhibited increased ss-SOFA scores compared to the conscious scores (Figure 1B), despite receiving a lower pathogen dose (Supplementary Information 3, Figure S1), having a lower amount of live pathogen in the blood measured by blood culture (Figure 1C), and exhibiting similarly high levels of pathogen-associated molecular patterns (PAMPS) (Figure 1D). This higher ss-SOFA in the anesthetized animals appears to be due to the cardio-depressive effects of prolonged general anesthesia lowering mean arterial pressure (MAP) in both anesthetized groups, with and without pathogen (Figure 1E). Lactate increased to the same degree in both models; however, it was cleared from the conscious animals while it remained elevated in the anesthetized animals (Figure 1F). Increased lactate and decreased MAP in the anesthetized model required that 3 out of 6 animals received epinephrine to maintain arterial pressure. As a result, these 3 animals were categorized with septic shock in accordance with the ss-Sepsis-3 criteria.

Following initiation of *E. coli* infusion, fever quickly developed in the conscious animals, peaking between 40 – 41°C by the end of infusion (Figure 1G). The temperatures of the anesthetized animals fluctuated initially, then remained constant between 37 – 38°C (Figure 1G). White blood cells and C-reactive protein showed no correlation to any stage of disease progression (Supplementary Information 3, Figure S2 and S3).

Analysis of the individual organ ss-SOFA scores revealed that increases in the total ss-SOFA were primarily driven by renal and blood coagulation dysfunction in both models, with cardiovascular dysfunction only observed in the anesthetized animals (Figure 2A). Importantly, the amount of pathogen in the organs did not correlate with the degree of organ dysfunction (Figure 2B, Supplementary Tables 1, 2 and Supplementary Information 4). For example, the liver had a high pathogen load in the conscious model compared to the anesthetized model; (Figure 2B and Supplementary Tables 1 and 2); however, the levels of hepatic organ dysfunction were both minimal (Figure 2A) in the time frame measured.

The highest organ ss-SOFA scores in both models were due to renal and coagulation effects (Figure 2A). Kidney dysfunction results in acute kidney injury (AKI) in human patients with sepsis [15], and thus, we measured AKI by quantifying percentage increases in CRE and categorizing them using the human Risk, Injury, Failure, Loss of kidney function and End-stage kidney disease (RIFLE) system. In the anesthetized model, only one animal developed kidney injury (Figure 3A), whereas 4 out of 6 animals developed AKI in the conscious model, 2 of which recovered to the risk category and 2 developed kidney failure (Figure 3B). The blood coagulation ss-SOFA scores increased

### Table 1: Swine-specific Sequential (Sepsis-Related) Organ Failure Assessment Score (ss-SOFA).

<table>
<thead>
<tr>
<th>System (measurement)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiration (PaO2/FiO2 mmHg)</strong></td>
<td>≥400</td>
<td>&lt;400</td>
<td>&lt;300</td>
<td>&lt;200</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>≥ 400</td>
<td>&lt;400</td>
<td>&lt;300</td>
<td>&lt;200</td>
<td>&lt;100</td>
</tr>
<tr>
<td><strong>Coagulation (Platelet Count)</strong></td>
<td>100 – 80% of baseline</td>
<td>67 - 80% of baseline</td>
<td>33 - 67% of baseline</td>
<td>&lt;33 % of baseline</td>
<td>&lt;13 % of baseline</td>
</tr>
<tr>
<td></td>
<td>≥150</td>
<td>&lt;150</td>
<td>&lt;100</td>
<td>&lt;200</td>
<td>&lt;100 with respiratory support</td>
</tr>
<tr>
<td><strong>Liver (Bilirubin mg/dl)</strong></td>
<td>0.3–0.6</td>
<td>0.7–2.0</td>
<td>2.1–5</td>
<td>5–10</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>&lt;1.2</td>
<td>1.2–1.9</td>
<td>2.0–5.9</td>
<td>6.0–11.9</td>
<td>12</td>
</tr>
<tr>
<td><strong>Cardiovascular (MAP mmHg)</strong></td>
<td>≥70</td>
<td>60–70</td>
<td>&lt;70</td>
<td>50–59 (epinephrine)</td>
<td>40–49 (epinephrine)</td>
</tr>
<tr>
<td></td>
<td>&gt;70</td>
<td>≤70</td>
<td>50–59 (epinephrine)</td>
<td>Dopamine &lt;5 or dobutamine (any dose)</td>
<td>Dopamine 5.1-15 or epinephrine &lt;0.1 or norepinephrine &lt;0.1</td>
</tr>
<tr>
<td><strong>Renal (Creatinine mg/dl)</strong></td>
<td>0.3–1.4</td>
<td>1.5–2.4</td>
<td>2.5–3.4</td>
<td>3.5–4.9</td>
<td>&gt;5</td>
</tr>
<tr>
<td></td>
<td>&lt;1.2</td>
<td>1.2–1.9</td>
<td>2.0–3.4</td>
<td>3.5–4.9</td>
<td>5</td>
</tr>
</tbody>
</table>

Arterial partial pressure of oxygen dissolved in the plasma (PaO₂). Fraction of inspired oxygen (FiO₂). Mean arterial pressure (MAP). Swine-specific ranges in bold font. Human ranges in regular font, from the Sepsis-3 criteria [5] for comparison (with the omission of Glasgow Coma Scale and alternate human measurements).

Figure 1: Sepsis model in swine with IV administration of a clinical, multi-drug resistant *E. coli* isolate.  
(A) Timeline of the anesthetized (n=6) or conscious (n=6) model of sepsis established in Yorkshire swine (n=4 or 2 controls for each model, respectively).  
(B) Swine-specific Sequential (Sepsis-related) Organ Failure Assessment (ss-SOFA) score for anesthetized and conscious models, evaluated using the ss-SOFA criteria outlined in Table 1, scored out of a total of 25.  
(C) Graph showing blood culture positivity over experimental time measured by the time to positive (TTP) in a BD Bactec system (growth of pathogen to 10^8 CFU/ml) and plotted as 1/TTP.  
(D) Graph showing Pathogen Associated Molecular Patterns (PAMPs) in whole blood as measured by Fc-Mannose Binding Lectin (FcMBL) in an Enzyme-Linked Lectin-Sorbent Assay (ELLeCSA) [14].  
(E) Mean Arterial Pressure (MAP) in mm Hg, measured by an invasive arterial pressure monitor showed hypotension in the anesthetized model compared to the conscious model.  
(F) Lactate measured by automated blood chemistry analysis remained high in the anesthetized model and was cleared in the conscious model.  
(G) Graph showing that temperature elevation was only observed in the conscious model, whereas a hypothermic response was observed in the anesthetized model. In all graphs, the anesthetized model with pathogen infusion is a grey solid line, anesthetized model without pathogen is a grey dotted line, conscious model with pathogen infusion is a black solid line and the conscious controls without pathogen infusion is a black dotted line.
to between 2 to 3 in the anesthetized model (Figure 3C) and 2 to 4 in the conscious model (Figure 3D). Interestingly, increased variability was observed in renal dysfunction between individual animals in the conscious group compared to coagulation dysfunction (Figure 3B vs. 3D), potentially indicative of inter-animal differences in tolerance to the pathogen. Temporally varied cytokine profiles were observed in both models (Figure 4). TNF-α peaked early in both models (Figure 4A), similar to IL-8 (Figure 4B). IL-6 peaked early and decreased more slowly in the conscious model (Figure 4C). In contrast, IL-1β peaked slightly later (~8 hours) in the conscious animals, with a lower level in the anesthetized model (Figure 4D).

**Figure 2:** Organ dysfunction.
(A) Graph showing the ss-SOFA score for each organ system at the end of the study (8 hours for anesthetized, pathogen-infused animals, light grey outlined bars, and 24 hours for conscious, pathogen infused animals, dark grey/black outlined bars). Organ systems evaluated included renal (Ren), blood coagulation (Coag), hepatic (Hep), cardiovascular (Card) and respiratory (Resp). (B) Graph showing the percentage of organ sections that contained pathogen in the kidney, liver, spleen, and lungs in the anesthetized (light grey outlined bars) and conscious (dark grey/black outlined bars) pathogen-infused animals.

**Figure 3:** Acute Kidney Injury (AKI).
(A and B) Kidney health classified using the Risk, Injury, Failure Loss of kidney function and End-stage kidney disease (RIFLE) system for individual anesthetized animals A-F. (A) and conscious animals G-L (B). (C and D), Blood coagulation ss-SOFA scores for individual anesthetized animals A-F (C), and conscious animals G-L (D).

**Discussion**
Sepsis is a clinically diagnosed disease that causes organ deterioration over time, mainly affecting elderly patients with multiple comorbidities. Therefore, if animal models of sepsis were to fully replicate the human disease, their time and expense would be prohibitive. Our goal was not to solve all the issues related to replicating a complex human disease in an animal model, but rather to rethink the way we compare data, symptoms, and outcomes in animals versus humans by establishing standardized evaluation criteria for a large animal model of sepsis. In this way, translation of pre-clinical data to human trials could...
Figure 4: Cytokine Responses. (A-F) Cytokine levels over time in anesthetized and conscious animals from EDTA plasma. Tumor Necrosis Factor-alpha (TNF-alpha) (A), Interleukin-8 (IL-8) (B), Interleukin-6 (IL-6) (C) and Interleukin-1 beta (IL-1 beta) (D). In all graphs, the anesthetized model with pathogen infusion is a grey solid line, anesthetized model without pathogen is a grey dotted line, conscious model with pathogen infusion is a black solid line and the conscious controls without pathogen infusion is a black dotted line.

Anesthetized compared to conscious animals had no fever and more severe cardiovascular dysfunction, resulting in septic shock in 3/6 animals. The lack of fever in these animals is consistent with the known hypothermic effects of anesthesia [8,9]. Cardiovascular dysfunction in the anesthetized model correlates with other anesthetized models of sepsis in swine with intraperitoneal or intravenous E. coli administration resulting in decreased MAP for the duration of the studies [16,17,23] with some also reporting increased lactate levels [24-26]. A lack of cardiovascular dysfunction has also previously been reported with sepsis induced in conscious swine following intravenous administration of endotoxin or E. coli [18,19]. The sustained increased lactate in anesthetized animals is potentially due to the combination of the pathogen and anesthesia causing prolonged hypotension resulting in hypoperfusion and tissue damage. The anesthetized control animals had a similar decrease in MAP compared to the pathogen challenged group; however, they did not develop hyperlactaemia. This suggests that the classification of septic shock in these animals was due to the combined effects of anesthesia and the response to pathogen. Pathogen-challenged conscious animals demonstrated similar hyperlactaemia as the pathogen-challenged anesthetized animals, however, the conscious group did not decrease MAP or require vasopressors, and they were therefore not classified as having septic shock. Here, by directly comparing the effects of infection using the same pathogen in anesthetized versus conscious animals, we can confirm that these cardiovascular differences are due to the combined effects of the general anesthesia and pathogen insult. This highlights the need to interpret animal data with care depending on the parameters of the experimental design.

Acute renal failure occurs in 19-50% of septic patients and this can lead to mortality rates as high as 60-80%, compared to 45% mortality among patients with acute renal failure alone [15]. Thus, one of our most important and clinically relevant findings is that acute kidney injury (AKI) was produced in both of our swine sepsis models, with RIFLE criteria for failure detected in 2 of 6 of the conscious animals.

Large animal models of sepsis induced by E. coli or endotoxin in anesthetized [16,17] and conscious [18,19] animals generally lack the breadth of clinical assessment to accurately determine the extent of overall organ failure. Sepsis-3 criteria allow clinical, real-time evaluation of multi-system organ failure severity to diagnose sepsis in patients. We modified these criteria to generate a swine-specific ss-Sepsis-3 system, including ss-SOFA scoring criteria, to evaluate disease progression in pigs. Our studies demonstrate the modified ss-Sepsis-3 criteria are useful for assessing and tracking disease progression in pathogen-infected swine. To our knowledge, this is the first demonstration of real time, clinical assessment of animal model sepsis symptoms using species-specific Sepsis-3-based criteria. This allows disease progression to be compared across animal models, species and to human clinical settings.

In this study, we used these standardized swine-specific evaluation criteria to track disease severity and progression in two models of sepsis involving either anesthetized or conscious animals that were infected with the same clinical isolate of E. coli pathogen, which was originally isolated from a septic patient. Anesthetized models allow multiple parameters to be measured with invasive equipment with relatively low costs due to their short timeframe and manageable number of personnel. The drawbacks of anesthetized models are that the anesthesia can adversely affect disease progression by aggravating symptoms, alter the sensitivity to infection or impair the animals’ response or measurements. Conscious models allow for longer study durations and clinical assessment of the animals by their activity, menace response, appetite, hydration and appearance [19-22]. However, the increased monitoring and time frame mean that these studies are more-costly and require increased personnel.

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This was not observed in the anesthetized model, likely due to the short time frame of the study and inadequate time for build-up of kidney damage indicators. Further sepsis progression may have occurred in the anesthetized animals if the study continued for longer times, but it was concluded earlier in the anesthetized group due to overwhelming cardiovascular issues, which were likely due to the combination of pathogen and anesthesia effects. The variability in individual animal kidney responses in the conscious model is consistent with the heterogeneous nature of organ damage seen in sepsis, especially in septic AKI, both in experimental models and clinically [27]. As the animals were outbred, genetic diversity may influence the individual animal response to a common stimulus or insult. Thus, an increased number of animals and measurements for renal blood flow and histopathological evaluation of acute tubular necrosis and apoptosis would be required to determine the significance of this variability in response [21].

Therapeutics for sepsis often target immune regulators such as cytokines and coagulation factors [28]. Cytokine expression was as predicted from literature, for example, showing early increases in TNF-alpha [29]. However, each cytokine also showed a distinct profile in these models, as is observed in patients with sepsis [30]. These temporally specific cytokine profiles and individual animal responses confirm that any potential therapies need to be carefully evaluated. It also raises the possibility to predict which animals will develop acute kidney injury or determine mechanisms conferring tolerance to infection in individual animals through computational modeling and multi-omics analysis.

Our modified ss-Sepsis-3 system and ss-SOFA scoring approach provide the foundation for quantifying the severity of future animal models with extended time frames, antibiotic therapy and intervention to more closely mimic the human condition [31]. Other species-specific modifications of the Sepsis-3 scoring system could be developed (e.g., dogs, rabbits, sheep), which might make longer-term experiments feasible.

Study Limitations

We recognize that this study has several limitations. Firstly, we used healthy, juvenile swine, without antibiotic treatment, lacking comorbidities commonly associated with human septic patients, such as cancer and diabetes. More expensive, adult Yucatan swine, with standard of care antibiotics, and more complex models with comorbidities could be introduced to confront this challenge.

A single pathogenetic agent was administered for both the anesthetized and conscious models with 6 replicates per group. To further establish the significance and robustness of this approach, a greater number of animals and other human-relevant pathogens, such as S. aureus or P. aeruginosa, and routes of administration and sepsis models should be evaluated. Polymicrobial infection models also could be utilized and assessed for longer timeframes [32].

We did not include quick SOFA in this study, as we did not measure respiration as breaths/minute or mental status; however, this could be pursued in future studies to determine its relevance for animal studies. A swine-specific Glasgow Coma Score (GCS) could also be introduced into this ss-SOFA scoring system to cover the full breadth of organ dysfunction. Given the blood coagulation dysfunction observed, markers of disseminated intravascular coagulation could be evaluated to provide additional endpoints. Finally, as information on normal ranges of clinical markers are limited for certain animal species or strains, further studies are needed to more accurately define the relevant ranges of blood marker values for species-specific SOFA scores.

Conclusions

A swine-specific Sepsis-3 evaluation system, including modified SOFA scoring, was developed to track sepsis progression in anesthetized and conscious pigs infected with a clinically relevant strain of E. coli pathogen. This is the first use of human equivalent sepsis scoring criteria, standardized for a particular animal species, for pre-clinical evaluation of sepsis disease progression. The use of this scoring system allows for a clinically relevant endpoint e.g. AKI, to be evaluated, which obviates the need to use mortality as an endpoint.

This first demonstration of a direct comparison of clinical manifestations of sepsis in anesthetized versus conscious animals using the same pathogen also revealed that the effects of anesthesia and model time frame can drastically affect organ dysfunction evaluation. Therefore, to avoid misinterpretation of results, animal model design parameters should be taken into consideration when evaluating experimental models of sepsis, and when applying this scoring system. The species-specific Sepsis-3 scoring system provides a foundation that can be applied to standardize evaluation criteria, compare results obtained between different models of sepsis and between animals and humans, which will hopefully improve the process by which novel sepsis therapeutics are developed, validated and translated into human clinical medicine in the future.

Acknowledgments

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Author Contributions

A.W., D.C.L., M.S., A.P.N. and D.E.I. designed the research, A.W., D.C.L., D.E.B., S.L., N.D., K.L., K.S., J.B.B., S.J., K.D. and A.P.N. performed and analyzed in vivo research, M.J.C., B.S. and P.L. designed, performed and analyzed microbiological experiments, A.W., D.C.L., M.S., A.P.N. and D.E.I. and wrote the manuscript with input from all authors.

Potential Conflicts

D.E.I. and M.S. hold equity in Opsonix Inc., and are consultants and members of its scientific advisory board. M.J.C. consults to the company. D.E.I. holds equity in SlipChips Corp. and is a member of its scientific advisory board; D.E.I and D.C.L. hold equity in FreeFlow Medical Devices Inc. and consult to the company.

References

Supplementary Information 1-4

Supplementary Information 1

Animal handling and surgical procedure

Animals were acclimatized for a minimum of 72 hours and housed in adjacent pens (20.7 square feet with a 12-hour light cycle from 7am to 7pm). Animals had food withheld for 12 hours prior to the study and free access to water prior to and throughout the experiment. Animals (in pairs, staggered by approximately 2 hours) were anesthetized with intramuscular injections of atropine (0.04 mg/kg), telazol (4.4 mg/kg) and xylazine (2.2 mg/kg) and maintained on isoflurane (1.5–3.0%) and oxygen (1.4-2.0 liter/min) delivered through an 8-9-mm endotracheal tube using a positive pressure ventilator. Animals were placed in the supine position and a 20-g intravenous cannula was placed in the left or right marginal ear vein for administration of drugs and fluids, and sterile saline (0.9% NaCl) was administered continuously at a rate of 100-400 ml/hr. A 12-Fr Foley catheter (SurgiVet, Smiths Medical) was placed for urinary drainage, and a 6 to 9 Fr percutaneous sheath catheter (Arrow, Teleflex, NC) was placed in the left or right femoral vein for pressure monitoring, while obtaining a blood sample before surgery to serve as a baseline.

Ventilator settings were determined on an individual basis depending on the pig size. When on mechanical ventilation, they were given a tidal volume of 10 ml/kg and a set respiratory rate; tidal volumes and respiratory rates were adjusted to keep the peak airway pressure between 12-18 mm Hg. Manual breaths were given at various time points throughout anesthetic procedure to allow expansion of the lungs and to prevent any lung collapse. End tidal CO$_2$ was monitored continuously and adjustments were made to the ventilation settings when these CO$_2$ levels got too low or too high (ideal range 30-40 mmHg). Blood gas values were also used to ensure ventilation settings were providing adequate support.

The right and left external jugular veins were exposed by bilateral neck cut downs, isolated, and cannulated with a 10 or 12 Fr sheath catheter (Cook Medical, Bloomington, IN) in the anesthetized animals or an 8 Fr sheath catheter (Arrow) in the conscious animals. Following cannulation, the neck incisions were closed in the anesthetized animals and heparin was administered at a constant rate (75-150 U/Kg/hr) using a syringe pump (Smiths Medical, Dublin, OH) to maintain an activated clotting time of at least 450 seconds. In the conscious animals, the right carotid artery was used for pressure monitoring for the rest of the procedure. The lines were tunneled approximately 2 inches from the incision site to make the lines more accessible for blood draws and monitoring. The neck incisions were closed and the femoral venous catheter was removed and pressure was applied for at least 15 minutes. Bupivacaine (Hospira, IL) was administered subcutaneously at the catheter sites and tunnel sites, a transdermal fentanyl patch was placed on the animal’s posterior, and buprenorphine (Reckitt Benekiser Healthcare (UK) Ltd., Hull, England) was administered intramuscularly. Animals were then dressed in a custom jacket (Lomir Biomedical Inc, Canada) to secure the lines, exubated and returned to housing for recovery. Animals were given free access to food and water, and plastic toys as enrichment.

Pathogen preparation

Bacteria were grown, prepared and tested prior to infusion, as previously described [1]. Briefly, *E. coli* 41949 (clinical isolate 41949 obtained from Brigham and Women’s Hospital, Crimson Biorepository, USA) were grown in RPMI media (Thermo Fisher Scientific, Waltham, MA, USA) containing 10 mM glucose to a 0.5 McFarland standard (Becton Dickinson, USA). Evaluation including plating to determine colony forming units (CFU), live/dead staining using BacLight (Thermo Fisher, USA), Bactiter™ (Promega, MI, USA), antibiotic susceptibility (minimum inhibitory concentration) and endotoxin content quantification using an Endoscan™ (Charles River Labs, USA). Serotype evaluation determined that *E. coli* 41949 tested positive for multiple O antigens and H26. It is not enterohaemorrhagic as it tested negative for shiga-type toxins 1 and 2. The strain also tested negative for heat-labile toxin, heat-stable toxin, cytoxic necrotizing factor 1 & 2 and intimin-gamma.

Pathogen infusion

For the anesthetized model, following closure of the incisions, *E. coli* was infused through the left external jugular catheter at a constant rate of 1.5-5.5 ml/hr for 2.75-4.3 hours with a final dose of 1.8-3.4 x 10$^5$ CFU/kg using a syringe pump (Smiths Medical, Dublin, OH). For the conscious model, following tunnelling and closure of the incisions, an initial bolus of *E. coli* was administered through the femoral vein with a syringe pump (1 x 10$^5$ CFU/kg except for one animal, which received half that concentration). Following recovery for 1.5 to 3 hours, a second infusion of pathogen was initiated for approximately 10 hours (1 x 10$^5$ CFU/kg) through the jugular vein. An intravenous infusion was chosen rather than an intraperitoneal bolus due to variability of systemic infection onset with the intraperitoneal route. The time difference of infusion for the anesthetized versus conscious groups was due to the concurrent hypotensive effect of anesthesia and pathogen insult in those animals.

Monitoring and blood sampling

Blood samples were obtained at intervals to carry out multiple measurements. Time to blood culture positive was determined from blood cultures using a BACTEC FX40 automated system with BD BACTEC Standard/10 Aerobic/F Culture Vials (Becton Dickinson, Sparks, MD, USA). Time to positive (TTP) measures the time required for bacteria in 10 ml of blood (taken from the animal into a blood culture vial) to grow to 10$^7$ CFU; the longer the TTP, the lower the blood pathogen load at the time of blood draw. Therefore, we presented these data as 1/TTP to clearly represent this response: the larger the 1/TTP value, the higher the pathogen dose. Complete blood count (CBC) (from EDTA anticoagulated blood), blood chemistry (from heparinized blood) and clotting profiles (activated prothomboplastin time and partial thrombin time, from citrated blood) were conducted using a VetScan HM2, VetScan Vs2, and a VetScan VSpro Hematology System (Abaxis, Union City, CA). Activated clotting times were measured using a Hemochront automated machine (Accriva Diagnostics). EDTA plasma was stored at -80 °C in Costar tubes (Corning, Inc., NY) for further analysis after centrifugation for 15 minutes at 1000 g and snap freezing in a dry ice/alkohol bath. Citrate plasma was stored in the same way with an additional centrifugation of the plasma for 10 minutes at 15000 g. Blood was anticoagulated using vacutainers (BD) or S-Monovette tubes (Sarstedt).

Heart rate, pulse rate, arterial pressure, oxygen saturation, CO$_2$ level, temperature and respiratory rate were monitored at intervals throughout the experiment. Measurement was measured using an esophageal probe in the anesthetized animals, with a rectal thermometer in the conscious animals. The pathogen infusion was decreased or halted, or the animals were euthanized, based on consultation with veterinary staff, if the following symptoms were observed: temperature > 40.5 °C, hypotension (mean arterial pressure < or approaching 45 mm Hg), serum creatinine > 3 mg/dl or a rise of > 0.3 mg/dl in 3 hours, decreasing PaO$_2$ below 90 mmHg, or reduced activity, menace response or appetite scores.


Volume 1, Issue 1

PAMPs Assay

Pathogen associated molecular patterns (PAMPs) were detected using a recently described Enzyme-linked Lectin-Sorbent Assay (ELLecSA) that is based on the binding of an engineered version of the carbohydrate binding domain of the human blood opsonin, Mannose Binding Lectin, linked to the Fc IgG domain (FcMBL) [1]. Briefly, EDTA blood samples were heparinized, recalcified and incubated with FcMBL-coupled magnetic microbeads (MyOne Dynabeads; Invitrogen, USA) and collected and washed using a KingFisher Flex™ (Thermo Fisher Scientific, Waltham, MA, USA) automated magnetic collection device. Captured PAMPs were detected using horse radish peroxidase conjugated rhMBL and tetramethylbenzidine (Sino Biological, P.R. China), terminated with 1 M sulphuric acid and the optical density measured at 450 nm. PAMP units were obtained from the FcMBL-ELLecSA by comparison to a mannan standard curve where 1 PAMP unit was equivalent to 1 ng/ml mannan binding [1].

C-reactive protein detection

Plasma C-reactive protein (CRP) levels were detected from citrated or EDTA plasma using a porcine specific sandwich CRP enzyme-linked immunosorbent assay (ELISA) (E-5CRP, Immunology Consultants Laboratory, INC, OR). Samples were processed according to manufacturer’s instructions with the sample dilution adjusted (1:30000, 1:20000 or 1:10000) according to the optical density at 450 nm using a Synergy H1 Hybrid Reader (BioTek, VT).

Cytokine detection

Plasma cytokine levels were detected from EDTA or citrate plasma using a ProcartaPlex Multiplex Immunoassay, specifically for porcine chemokine and cytokines (Affymetrix Company; eBioscience, Vienna, Austria), by following the manufacturer’s instructions. A Bio-Rad Bio-Plex Pro II washer station (Bio-Rad Laboratories, Inc., Hercules, CA) was used for the wash steps, and a Bio-Plex 3D Suspension Array System with Luminex xMAP Technology (Bio-Rad Laboratories, Inc., Hercules, CA) was used for detection and quantification.

Necropsy

At the conclusion of each study, animals were heparinized (300 U/kg) and then they received a second dose of atropine (0.04 mg/kg), telazol (4.4 mg/kg) and xylazine (2.2 mg/kg) before an intravascular lethal dose of Fatal Plus. After the animals were euthanized, organ samples of lung, kidney, liver and spleen were collected under sterile conditions. The lung samples were collected via a sternotomy: a cut down was performed to expose the sternum, the sternum was removed, the pulmonary pleura was opened, and samples from the cephalic and caudal sections of the left and right lung were collected. The kidney, liver, and spleen samples were collected via laparotomy: a cut down was performed to open the abdominal cavity, a small incision into the peritoneum was made to expose the right kidney, the whole kidney was collected, and then specific sections were taken. Samples from each lobe of the liver and samples along the spleen were also surgically excised and collected.

Organ culture

Two 0.5-1 cm³ samples from 4 consistent, distinct locations per organ were placed into separate pre-sterilized and pre-weighed Nalgene bottles (Thermo Fisher Scientific, Waltham, MA, USA) containing stainless steel balls (McMaster-Carr, Robbinsville, NJ). Bottles containing organ pieces were weighed and 17 ml of sterile water was added. Organ pieces were fragmented by bead mill treatment at 30 Hz for 3 min using a Mixer Mill MM 400 machine (VerderMill, Inc., USA). CFUs were quantified as E. coli CFU/g from the resulting slurry by spiral plating (Eddy Jet 2, IUL, Barcelona, Spain), automated counting (Flash & Go, IUL, Barcelona, Spain) and adjusting for the volume of water added and the weight of the organ pieces. Single species identified to be E. coli were seen in all organ cultures from animals that received E. coli infusions. All control animals had no pathogen in the organ cultures except in the liver and kidney of one anesthetized control animal, identified as E. fergusonii, and in the kidney and spleen of a second anesthetized control animal, identified as S. chromogenes.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). This was a pilot study, therefore, hypothesis testing was not applicable in this context and no power analysis was conducted to determine sample sizes.

Supplementary Information 2

Swine-specific SOFA Scoring System

Respiratory ss-SOFA was measured using the same arterial oxygen partial pressure to fractional inspired oxygen (PaO₂/FiO₂) categories as for humans. Oxygen was provided to the anesthetized model animals with the isoflurane, and was provided to the conscious model animals via a facemask if blood oxygen saturation began to decrease.

Blood coagulation ss-SOFA was measured by converting the human SOFA platelet count (PLT) categories to a percentage of baseline platelet count for each individual animal because the normal platelet count in swine covers a broader range compared to humans.

Hepatic ss-SOFA categories were measured by converting the human SOFA platelet count (PLT) categories to a percentage of baseline platelet count for each individual animal because the normal platelet count in swine covers a broader range compared to humans.

Cardiovascular ss-SOFA was measured using mean arterial pressure (MAP) as it is in humans, with a MAP of 70 mm Hg as normal (SOFA = 0) and <70 mm Hg as SOFA = 1. Due to the cardio-depressive effects of isoflurane anesthesia, vasopressors (epinephrine) were administered with a MAP below 55 mm Hg. The ss-SOFA score increased if the MAP continued to drop when an increasing vasopressor dose was administered.

A renal ss-SOFA score was determined by measuring increased serum creatinine (CRE) levels. The renal ss-SOFA categories 0 – 2 were modified from the human values because in our experience Yorkshire swine displayed a larger normal range of CRE (0.3 – 1.4 mg/dL).

We did not include a CNS ss-SOFA score in this study because it could not be used with anesthetized animals, and although menace response and activity were measured in the conscious model, we believe a more comprehensive method for determination of neurological function to correlate with the human Glasgow Coma Score would be required. For example, although verbal response criteria are not appropriate for animals,
the use of the eye-opening score and best motor response score (or modifications thereof) could be applied to animals to create standardized criteria which could be used to compare conscious animal models.

Using this approach, we developed a ss-Sepsis-3 classification scheme with a maximum ss-SOFA score of 25 in which a ss-SOFA ≥ 2 was deemed an indicator of sepsis, with septic shock being defined as a ss-SOFA ≥ 2 with the need to administer vasopressors and increased lactate levels (a value of >2.5mM lactate was used to reflect the increased muscle to weight ratio of swine compared to humans).

**Supplementary Information 3**

**Supplementary Figure Legend**

Figure S1. Concentration of pathogen infused over time for the conscious (black area) and anesthetized (grey area) models.

**Supplementary Information 4**

**White blood cells and C-reactive protein**

Leukocytopenia was detected in both the conscious and anesthetized animals over the course of the initial 8 – 10 hours (Fig. S2). However, this lowering of white blood cell (WBC) number was followed by leukocytosis only in the conscious model, with levels rising to over $3 \times 10^9$ cells/L by 20 hours. Given the heterogeneous nature of sepsis progression, temporal fluctuations in both temperature and WBC count are common, and do not correlate with any particular stage of sepsis or organ dysfunction; therefore, these parameters are less useful as symptoms to consider when comparing animal and human responses in sepsis.

As expected, the commonly used inflammatory marker C-reactive protein (CRP) was elevated variably in all animals, even in animals that only received surgery (Fig. S3). Indeed, CRP elevation is not specific for infection as it is also elevated in acute sterile inflammation [2].

**Supplementary Figure and Table Legends**

**Figure S2.** Graph showing White Blood Cell (WBC) count over time, measured by complete blood count (CBC) and the development of leukocytosis in both models compared to controls. The anesthetized model with pathogen infusion is a grey solid line, anesthetized model without pathogen is a grey dotted line, conscious model with pathogen infusion is a black, solid line and the conscious controls without pathogen infusion is a black dotted line.

**Figure S3.** Graph showing C-Reactive Protein (CRP) as measured by ELISA from EDTA plasma. The anesthetized model with pathogen infusion is a grey solid line, anesthetized model without pathogen is a grey dotted line, conscious model with pathogen infusion is a black, solid line and the conscious controls without pathogen infusion is a black dotted line.

**Table S1.** Average pathogen load for each individual conscious pig. Table showing the colony forming units per gram of tissue (CFU/g) for lung, liver, spleen and kidney, as quantified by organ culture in each individual pig (numbered 1-6).

<table>
<thead>
<tr>
<th>Organ</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>113</td>
<td>226</td>
<td>1451</td>
<td>235000</td>
<td>2810</td>
<td>894</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>268</td>
<td>1256</td>
<td>2620</td>
<td>221</td>
<td>297</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>0</td>
<td>1091</td>
<td>2219</td>
<td>545</td>
<td>736</td>
</tr>
<tr>
<td>Spleen</td>
<td>138</td>
<td>1585</td>
<td>2636</td>
<td>8123</td>
<td>957</td>
<td>662</td>
</tr>
</tbody>
</table>

**Table S2.** Average pathogen load for each individual anesthetized pig. Table showing the colony forming units per gram of tissue (CFU/g) for lung, liver, spleen and kidney, as quantified by organ culture in each individual pig (numbered 1-6).

<table>
<thead>
<tr>
<th>Organ</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1324</td>
<td>227</td>
<td>0</td>
<td>1469</td>
<td>2583</td>
<td>245</td>
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<tr>
<td>Liver</td>
<td>64</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>183</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>75</td>
<td>98</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Supplementary Information 4

Hepatic dysfunction played a minor role in both models, despite there being a large pathogen load in the liver in conscious animals, similar to other organs (Fig. 2A,B). Mild respiratory dysfunction was detectable in the conscious model (Fig. 2B), however, in most animals the oxygen saturation of the blood remained high. This was not detected in the anesthetized model, despite the lungs having the largest pathogen load in the anesthetized animals (Fig. 2B). This was likely because the animals received inhaled pure oxygen with the isoflurane which masked or reduced the organ dysfunction. These results are consistent with the observation that PAMPs, including endotoxin, which are shed from live pathogens or contained within debris released by killed microbes, as well as the live pathogen, are responsible for triggering the inflammatory cascade that leads to multi-organ damage in patients with sepsis [3, 4].

Supplementary References
