University of Szeged

Albert Szent-Györgyi Medical School

Doctoral School of Multidisciplinary Medical Science

Bridging the gap between experimental and human sepsis:

Porcine models for sepsis research with improved clinical relevance

Ph.D. Thesis

Dr. Bettina Ágnes Zsikai

Supervisor: József Kaszaki Ph.D.

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1. INTRODUCTION

Sepsis remains a leading cause of mortality in intensive care units worldwide. The World Health Assembly (WHA) and World Health Organization (WHO) have made sepsis a global health priority and adopted a resolution to improve prevention, diagnosis, and management (Reinhart et al., 2017). In the last three decades, the concept of sepsis has developed continuously, with a large number of studies involving observations of septic patients and experimental modeling of the pathology.

1.1. Changes in definitions of sepsis over time

A modern definition of sepsis was first formulated by the American College of Chest Physicians and the Society of Critical Care Medicine at a consensus congress in 1991 (Sepsis-1), which provided classifications for severe sepsis, septic shock, and multiple organ dysfunction syndrome. At the consensus conference, sepsis was defined as the co-existence of infection and systemic inflammatory response syndrome (SIRS) (Bone et al., 1992). Definitions were updated in 2001, when sepsis was defined as a systemic inflammatory response to infection (Levy et al., 2003). In 2002, the Surviving Sepsis Campaign (SSC) was set up to reduce sepsis-related mortality. The SSC proposed a series of care bundles organized in a protocol of early and simple goals (Rello et al., 2017). In 2016, the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3) consensus conference redefined the terms inflammation, sepsis, and septic shock. According to the new definition, sepsis is a potentially life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock is a subcategory of sepsis, which includes more severe circulatory, metabolic, and cellular disorders than general sepsis, and is characterized by uncontrollable hypotension despite adequate fluid resuscitation (Singer et al., 2016). Clinical characteristics of human sepsis have been repeatedly standardized in recent decades, and today the Sequential (sepsis-related) Organ Failure Assessment (SOFA) scoring system adequately characterizes the level of dysfunction of vital organs. SOFA is based on six different scores, one for each of the respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems, each scored from 0 to 4 with an increasing score reflecting worsening organ dysfunction (Vincent et al., 1996). The Quick SOFA Score (qSOFA) was introduced by the Sepsis-3 group in 2016 as a simplified version of the SOFA score (Angus et al., 2016). It provides simple bedside criteria to identify adult patients with suspected infection, incorporating altered mentation, systolic blood pressure of 100 mm Hg or less, and a respiratory rate of 22/min or greater (Singer et al., 2016).

1.2. Pathomechanism of sepsis and septic shock

The pathogenesis of sepsis is complex and involves multiple aspects of the interaction between the infecting microorganisms and the host. The pathomechanism of sepsis is now considered to be an uncontrolled host response to infection, in which the imbalance between oxygen delivery (DO_2) and consumption (VO_2) and the decrease in oxygen extraction (ExO_2) at the cellular level play a key role. During the initial stage of infection, there is an overproduction of inflammatory mediators causing what is referred to as a "cytokine storm." This surge involves various factors like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 (IL-1), leading to fever and a state of increased metabolism and inflammation (Chousterman et al., 2017; Piechota et al., 2007). This condition raises both tissue and cellular oxygen consumption as well as oxygen extraction. Furthermore, the "cytokine storm" triggers the production of nitric oxide (NO), resulting in systemic vasodilation and a consecutive decrease in total peripheral resistance (TPR). To counteract these changes, compensatory mechanisms such as elevated heart rate, cardiac output (CO), and increased DO_2 help offset the reduced TPR and supply oxygen for the increased VO_2 (Armstrong et al., 2017). Loss of the vasodilator-vasoconstrictor balance, the decrease of adequate systemic filling pressure, and the hyper-coagulation result in insufficient microperfusion, which leads to tissue hypoxia (Ince et al., 2016). Inadequate microcirculation may result in a decrease in tissue and subcellular oxygen and substrate supply, which may determine mitochondrial oxygen consumption. A serious consequence of mitochondrial dysfunction is increased production of reactive oxygen derivatives and the resulting direct cellular damage caused by oxidative stress, which is believed to mediate the septic organ damage (Zhang et al., 2018). The progression may result in a hypodynamic phase of sepsis, characterized by the exhaustion of compensatory mechanisms, immunoparalysis, progressive fall in blood pressure and a decrease in TPR, which may indicate severe multi organ failure (MOF) (Armstrong et al., 2017; Singer et al., 2016).

1.3. Biomarkers of sepsis

A wide range of biomarkers is available for clinicians and researchers to aid in evaluating and managing sepsis. Toll-like receptors bind to pathogen-associated molecular patterns and initiate intracellular signaling cascades that result in the synthesis and release of proinflammatory biomarkers that regulate early responses, such as TNF-α, IL-1, IL-6, IL-8 and high-mobility group box protein-1 (HMGB1) (Rivers et al., 2013). At the same time, the production of anti-inflammatory biomarkers - such as interleukin 10 (IL-10) - is also started, which is important to prevent tissue damage, inhibit inflammation, and enhance healing. In cases of sepsis, these pro- and anti-inflammatory mechanisms are disrupted and overturned (Rittirsch et al., 2008;

Taeb et al., 2017). Big endothelin (bET) and HMGB1 were chosen as biomarkers of tissue hypoxia and necrosis (Chaudhry et al., 2013). Levels of plasma nitrite/nitrate (NO_x), stable end-products of nitric oxide (NO), are markers of nitric oxide synthase activity, and thus the production of nitric oxide radicals (Radi, 2018). Although lactate is not a sepsis-specific biomarker, elevated serum lactate levels may indicate severe sepsis, imply progression to organ dysfunction, and are associated with an elevated mortality rate (Rello et al., 2017).

1.4. Preclinical sepsis modeling according to evidence-based guidelines

A prerequisite for a detailed understanding of the pathomechanism is appropriate experimental modeling. Animal models that faithfully reproduce the complex pathology and are suitable for clinical translation are essential to identify new therapeutic targets for sepsis and septic shock, thus reducing mortality. The recently established Minimum Quality Threshold in Preclinical Sepsis Studies (MQTiPSS) recommendations for study designs enable us to standardize experimental sepsis, including the assessment of organ failure/dysfunction parameters, which reflect the specificities of the human disease (Osuchowski et al., 2018). They highlighted the importance of subsequent evaluation of established signs of organ failure, similarly to the SOFA scoring systems in human patients (Singer et al., 2016; Vincent et al., 1996). Although these points are made for general purposes, they apply more to rodents (rats and mice) than to larger laboratory animals (pigs or sheep). Porcine models of sepsis, however, offer many advantages over rodent studies, as domestic pigs are more closely related to humans in terms of anatomy, genetics, and physiology (Swindle, 2012; Meurens, 2012). Furthermore, pigs are more suitable for clinically-relevant anesthesia, instrumentation, and intensive care, including invasive hemodynamic monitoring, fluid resuscitation, and repetitive blood sampling (Guillon, 2019). These characteristics allow for a more standardized induction and individual evaluation of disease progression and severity, but the need for anesthesia and observation time are still restrictive or limiting factors (Soerensen, 2012; Park, 2019; Waterhouse, 2018).

2. MAIN GOALS

Our objective was to improve the design of swine models of sepsis taking into account the above considerations, with the final goal being to reduce heterogeneity and the gap between the messages of animal and human studies. Initially, we followed the Sepsis-1 definitions and designed our experimental setup accordingly in Study 1 (Érces et al., 2011; Zsikai et al., 2012). Later, we modified our model according to the Sepsis-3 criteria in Study 2 (Rutai et al, 2022). In this context, **our aims** were:

In Study 1:

I. To create a standardized **experimental protocol** that can take advantage of previously presented studies and is compatible with the clinical course of human sepsis. Sepsis will be induced by polymicrobial live pathogens, whereas the clinically relevant progression phase will be carried out in the awake state without intervention.

II. To design a post-instrumentation **monitoring period** to establish a diagnosis of sepsis according to Sepsis-1 criteria and to measure the macro- and microcirculatory responses and biomarkers of tissue hypoxia.

In Study 2:

III. To develop and use a species-specific **pig SOFA scoring system** (pSOFA) based on the human scoring system, which provides real information on the degree of organ damage but with modifications to take the physiology of the pig into account.

IV. To demonstrate the importance of **microbiological analysis** of intraperitoneally injected fecal inoculum or blood cultures, with an examination of a correlation between degree of microbiological insult and severity of sepsis/organ damage.

V. To detect how early elevation and dynamics of dedicated **inflammatory markers** are associated with sepsis severity and outcome.

3. MATERIALS AND METHODS

3.1. Experimental protocols

The experiments were performed on Vietnamese minipigs of both sexes. In both studies, the animals were randomly allocated into control (sham-operated; Study 1 n = 6; Study 2 n = 9) and septic groups (Study 1 n = 9; Study 2 n = 27). The experimental protocol for both studies was divided into four stages.

3.1.1. Stage 1: Baseline measurements and blood sampling under temporary anesthesia

After anesthesia, a permanent central venous catheter with three lumina was introduced into the jugular vein for fluid therapy and blood sampling (t=0). Endotracheal intubation was performed, and the animals were ventilated mechanically. Basic ventilation settings (respiratory rate (RR): 10–12/min; tidal volume (TV): 8 mL/kg; positive end-expiratory pressure (PEEP): 4–5 cmH_2O; fraction of inspired oxygen (FiO_2): 21%) were checked by pulse oximetry using a sensor fitted to the tongue of the animals. The respiratory rate was adjusted to maintain the end-tidal carbon dioxide pressure (controlled by capnometry) and the partial arterial carbon dioxide pressure ($PaCO_2$) within a range of 35–45 mm Hg.

3.1.2. Stage 2: Sepsis induction

Polymicrobial autologous fecal induction inoculum was injected into the intraperitoneal space using a blunt-pointed 12G Veress needle at t = 0 h. The control animals were treated with 200 mL of sterile saline in the same manner. After induction, the animals were extubated and awakened with a gradual reduction of anesthesia and mechanical ventilation.

3.1.3. Stage 3: Sepsis progression

The spontaneously breathing animals were placed in a test cage and a blood sample was taken 6 hours after sepsis induction. The animals received 15 mL/kg/h crystalloid (Ringerfundin®) iv at hours 6 and 12 after sepsis induction to maintain fluid balance, while analgesia was performed with nalbuphine iv (0.2 mg/kg) through the jugular vein.

3.1.4. Stage 4: Invasive hemodynamic monitoring, sampling, and severity scoring

Invasive hemodynamic monitoring was started 15 hours after sepsis induction. The animals were re-anesthetized. Mechanical ventilation was started after reintubation. A urinary catheter was placed surgically in the bladder to measure hour diuresis. A thermistor-tip transpulmonary thermodilution catheter was placed in the right femoral artery for invasive hemodynamic monitoring (cardiac output, cardiac index, extravascular lung water index, heart rate, mean arterial pressure) and core temperature measurement. In Study 1, a pulmonary artery catheter was also introduced via the femoral vein by tracing the pulmonary pressure signals, and a silastic balloon-free tonometric probe was introduced into the small intestine to monitor intramucosal pCO_2 levels by capnometry (Boda et al., 2006). $PaCO_2$ levels were taken simultaneously and subtracted from the tonometric pCO_2 levels to calculate the pCO_2 gap values. Hemodynamic measurements, blood gas analysis, and, in Study 2, the assessment of organ failure were performed hourly between hours 16 and 24 of the experiments. Blood gases were analyzed simultaneously every hour with a cooximetry blood gas analyzer. Oxygen delivery, oxygen consumption and oxygen extraction values were calculated. The degree of respiratory failure was determined with the PaO_2/FiO_2 ratio. The systemic vascular resistance index (SVRI) and stroke volume index (SVI) were calculated according to standard formulas. The pSOFA scoring in Study 2 was done between hours 16 and 24. Based on the consensus criteria of the Sepsis-3 definitions (sepsis involves proven organ damage, with a SOFA score $\geq$ 2; lactate level $\geq$ 2; septic shock is sepsis with persistent hypotension, requiring vasopressors to maintain MAP $\geq$ 65 mmHg despite adequate fluid resuscitation (Singer et al., 2016)) the septic animals were allocated into two subgroups, a sepsis (n = 10) and a septic shock group (n = 9), 18 hours after induction.

3.2. Intravital videomicroscopy of the microcirculation

In order to study the microcirculation we used intravital videomicroscopy at t = 0, 16, 20 and 24 h. In Study 1, the intravital orthogonal polarization spectral (OPS) imaging technique was used, while in Study 2, the Incident Dark Field (IDF) imaging technique was used for a non-invasive examination of the sublingual microcirculation. The proportion of perfused vessels (PPV) was calculated as the ratio of the length of all detected vessels to the length of vessels with measurable flow values (percentage) (Dobbe et al., 2008; Massey et al., 2016).

3.3. Detection of organ functions: Inflammatory and metabolic markers and blood cell count

Plasma nitrite/nitrate (NO_x) were determined in both studies. NO_x were measured with the Griess reaction. The assay depends on the enzymatic reduction of nitrate to nitrite. Plasma levels of TNF-α and IL-10 as well as bET and HMGB1 were determined from these samples using commercial ELISA kits according to manufacturer's instructions. Keeping pace with Sepsis-3 and the SOFA scoring system, liver dysfunction was assessed by measuring plasma bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, whereas the extent of kidney injury was estimated by measuring plasma creatinine and albumin levels. The lactate level as an indicator of metabolic imbalance was measured from venous blood samples. In Study 1, only the white blood cell count was tested as a marker of inflammation, using conventional Bürker's chambers. In Study 2, samples for platelet, white blood cell, and red blood cell counts were placed in EDTA-coated tubes and analyzed within four hours with an automated cell counter.

3.4. Blood culture analysis

Blood samples were obtained from 14 randomly selected animals in the septic group at hour 18 of the experiment in Study 2 and then transferred to aerobic and anaerobic media bottles. The microbial composition of the inoculum was analyzed for the most frequent species by MALDI-TOF mass spectrometry (MS; Bruker Daltonics, Germany)

3.5. Calculation of pSOFA scores

In Study 2, similarly to the human SOFA and quick SOFA scores, we established two scoring systems for the pigs consisting of three or five domains of organ/organ system dysfunction parameters. As a respiratory parameter, the PaO_2/FiO_2 ratio was used with threshold values similar to those in humans. During 3D- or 5D-pSOFA scoring, urine output was used as an indicator of renal dysfunction. Since pigs display a broader platelet count range than humans, values up to 200×10^9/L were considered normal (Waterhouse et al., 2018). We used the same threshold values for bilirubin as those used in human SOFA scoring.

3.6. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows). Normality of data distribution was analyzed with the Shapiro–Wilk test. The Friedman analysis of variance on ranks was used within groups. Time-dependent differences from the baseline for each group were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method; p values < 0.05 were considered significant. Correlations between two variables were examined using the Spearman rank correlation coefficient.

4. RESULTS

4.1. Parameters of the SIRS criteria in Study 1

In the septic group, the **heart rate** increased constantly and exceeded the control values significantly. **Arterial partial carbon dioxide tension ($PaCO_2$)** already showed a significant decrease from hour 16 compared to the control group. Growth in **blood temperature** was observed as the inflammatory process progressed, which was significantly higher from hour 20 compared to the control group, and a significant rise from baseline was also found from hour 21. The drop in **white blood cell** count was already significant at hour 18 compared to the constant data of the control group. We noted significant leukopenia in our septic animals from hour 20. The control group results did not demonstrate changes according to the SIRS criteria.

4.2. Macrohemodynamic changes in Study 1

MAP gradually decreased below 70 mmHg in the septic animals, while the **cardiac index** was remarkably elevated and surpassed the control level significantly from hour 20. In the septic group, the **SVR** started to fall at hour 18 of the experiments and reached the deeper point at hour 20; then the values were maintained at this low level until the end of the study. In the septic group, **ELWI** already showed a significant difference at hour 16 compared to the control group and continued to deteriorate during the experiment.

4.3. Direct and indirect microcirculatory changes in Study 1

The **pCO_2 gap** of the small intestine increased significantly in the septic group at the beginning of invasive monitoring and remained significantly higher than that for the sham-operated control group. At about hour 20, the pCO_2 gap showed a sudden rise. A direct analysis of the sublingual microcirculation revealed a gradually decreasing and statistically significantly lower **red blood cell velocity** and **capillary perfusion rate** in the septic group as compared to both baseline values and the control group from hour 16 of the experiment.

4.4. Changes in plasma biomarkers in Study 1

Sepsis induction resulted in a statistically significant rise in **NO_x** level as compared to the baseline values and to the control group, which was already visible in the early hours. The plasma **HMGB1** concentration in the septic group gradually grew and reached a significant, approximately five-fold increase by hour 16 after the induction of sepsis compared to the control group. Afterwards, a subtle decrease was observed, which only showed a significant difference from the baseline value by hour 24. Plasma **bET** levels demonstrated a rapid, significant rise in sepsis. The growth peaked at hour 6 and remained high throughout compared to the control group.

4.5. Experimental sepsis model based on Sepsis-3 criteria: Study 2

In Study 2, the pSOFA scoring system was used to divide the animals into septic and septic shock subgroups in addition to the control group. In the septic shock group, significantly lower **MAP** values were seen than those in the sham-operated animals during the entire 8-hour observation period. A temporary hypotension also developed in the sepsis subgroup in the last 2 hours. **Urine output** values already showed a significantly lower value at hour 16 compared to the control group, and, in some cases, a significant difference was also seen compared to animals classified as septic by the pSOFA score. The **PaO_2/FiO_2 ratio** varied similarly in the two septic groups, but the drop became significant by the beginning of the instrumental phase in the septic shock group. A progressive fall in **platelet count** was observed in all groups during the last three hours of the study period, with the lowest values in the septic shock subgroup. As compared to the sham-operated animals, only the septic shock subgroup showed significant elevations in **plasma bilirubin** concentrations and in the **AST/ALT ratio**, which occurred during the last 3–4 hours of the study. **Plasma creatinine** levels were also higher in the septic shock subgroup than in the other groups. A progressive drop in **plasma albumin** levels was observed in all the groups.

4.5.1. Changes in pSOFA scores

In the septic shock subgroup, significantly higher **3D-pSOFA** and **5D-pSOFA** scores were detected than those in the sham-operated group and sepsis subgroup throughout the 8-hour observation period. A significant difference was observed between the sham-operated and septic groups at hour 24 after the sepsis induction for both scores.

4.5.2. Macrohemodynamic changes

In both sepsis-inoculated groups, a significant increase in **heart rate** was observed as compared to the control group during the entire examination period. In the septic shock group, **cardiac**

index deteriorated during the last two hours of the experiments. A significant decrease in **stroke volume index** was observed in both septic groups. The two septic groups are also well separated by the values for the **systemic vascular resistance index**. The septic shock group shows a significant reduction from hour 18 compared to the control group.

4.5.3. Microcirculatory changes

Both septic groups showed a significantly deteriorated microvascular perfusion (reduced **PPV** values) in the sublingual mucosa, which reached its minimum at hour 20 of the experiments. By hour 24 of the experiments, an improvement in PPV was observed.

4.5.4. Oxygen dynamics

Although no differences were observed in $\mathbf{DO_2}$ in the three groups, significantly increased $\mathbf{VO_2}$ values were measured in the septic shock subgroup at hours 18 and 24 compared to the sham-operated animals. Both sepsis-challenged groups showed a significantly elevated degree of **oxygen extraction** irrespective of the severity of sepsis.

4.5.5. Blood cell counts

White blood cell counts did not change in the sham-operated group, while significant leucopenia developed after 16 hours of sepsis induction in both sepsis-inoculated groups. The **red blood cell** counts did not change in any of the groups studied.

4.5.6. Microbial features of the inducer inoculum

The retrospective microbiological analysis showed that the microbial concentration of the inoculum ranged between 3.8×10^7 and 4.95×10^9 colony-forming units (CFU). Concentrations above 1.4×10^9 CFU increased the risk of septic shock, while values above 8×10^9 CFU resulted in devastating deterioration in animal well-being. The most common microorganisms found in the feces suspension were E. coli and Klebsiella pneumoniae, which generally cause Gram-negative sepsis in humans. The occurrence of E. coli was 100% in the fecal samples. We also found several Lactobacilli species.

4.5.7. Relation between microbiological concentrations and pSOFA scores

There was a moderate, significant positive correlation between the inoculum CFUs and the late, 24^{th}-hour 3D-pSOFA and the 5D-pSOFA score values. The concentration of microbes in the blood culture showed a strong, significant correlation with both the 18^{th}-hour 3D-pSOFA and the 5D-pSOFA score values.

4.5.8. Changes in plasma levels of inflammatory biomarkers

Similar **TNF-α** levels were detected in both septic groups. Plasma TNF-α concentration peaked at hour 6 after sepsis induction, and this significant elevation persisted during the entire period

of invasive hemodynamic monitoring in the septic shock subgroup. The plasma levels of **HMGB1** in the septic group only showed an increase compared to the baseline value, whereas a significant, approximately three-fold growth was observed in the septic shock group at hour 16 of the experiment. This marked rise displayed a decreasing trend towards the end of the observation period. Plasma levels of **IL-10** also showed an early significant increase and a peak at hour 16 in both septic groups compared to the control group and baseline values. This difference disappeared by hour 20. A change in plasma **bET** levels was only observed in the septic shock subgroup, with a significant increase seen throughout the instrumented phase, 16–24 hours post-inoculation. Plasma **NO_x** levels showed an increase in both septic groups. The difference was seen by hour 16, where the two septic groups were well separated by the NO_x levels. In the septic shock group, significantly higher NO_x levels were measured compared to the sham-operated group and baseline values. The whole blood **lactate** showed some degree of elevation at 6 hours after induction in both septic groups, but it only reached significantly higher values in the septic shock group, which persisted during the entire period.

4.5.9. Correlation between plasma biomarkers and pSOFA scores

We found significant correlations between the late, 24th-hour 3D- and 5D-pSOFA scores and the early, 6th-hour TNF-α, the 16th-hour bET, and the 16th-hour HMGB1 levels. However, we found no correlation between the 24th-hour 3D- or 5D-pSOFA scores and the 16th-hour IL-10 values.

5. DISCUSSION

5.1. Development of the experimental model suitable for clinical translation

Failure to transfer experimental results from animals to humans is mainly due to inadequate animal models that do not fully mimic human sepsis. This study presents a porcine model of polymicrobial, intraabdominal sepsis with clinically relevant hemodynamic responses, a laboratory profile, inflammatory biomarkers and bacteremia, in which our experimental protocol was designed according to the recommendations of the MQTiPSS guidelines.

In the **induction phase** of our experiments, it was possible to accurately standardize the intra-abdominal septic insult with a pre-defined amount of fecal inoculum and thus reproduce the inflammatory-hemodynamic responses. Improving on the model developed by Barth et al. (2008), we designed our Study 1 protocol so that invasive instrumentation and monitoring would start 15 hours after sepsis induction. Thus, the **progression phase** of our setup combines the advantages of conscious and anesthetized in vivo models and mimics human sepsis and multi-organ failure very closely, while adhering to ethical standards on the use of animals for

scientific purposes. In this model, organ-supportive therapies are used as in the ICUs: respiratory support, fluid resuscitation, and diuretic, vasopressor, and inotropic therapies. These individualized treatments began 16 hours after the insult in a goal-directed manner in accordance with the current condition of the animals and using recommendations similar to those for humans (Corrêa et al., 2012; Head LW et al., 2016). The improved clinical compatibility of the model also includes the fact that **invasive monitoring** is initiated at a later stage of the septic process, when there are already clear signs of infection, and that, in accordance with clinical practice, appropriate diagnostic and therapeutic steps are only performed afterwards. In our experiments, we were able to demonstrate the compensatory mechanisms that are typical of human sepsis. Our study demonstrated different patterns of microvascular alterations between septic and control animals in both intestinal and sublingual regions, and these data clearly indicated the presence of substantial intestinal and peripheral hypoxia in spite of the hyperdynamic macrocirculation. The microcirculatory reaction could be a consequence of the altered synthesis of NO and proinflammatory cytokines. Summarizing our data from Study 1, we found that the micro- and macrohemodynamic changes and other pathological features recorded in our model of minipigs are remarkably similar to the clinical signs detected in human sepsis. This peritonitis model characterizes human sepsis appropriately and can be applied in further research and in the development of new therapeutic approaches.

5.2. Evaluating sepsis according to the Sepsis-1 criteria

In our Study 1 setup, we did not yet use the SOFA score; therefore, although signs of organ damage were observed, they were not monitored. Our focus was on observing and describing the inflammatory process. Septic animals were only compared to control group animals that were classified as septic based on core temperature, heart rate, $PaCO_2$, and white blood cell count. With this evaluation, animals in the SIRS phase were included in the septic group. Non-responders were excluded from the study. Despite the considerable variance in our results, we found statistically significant differences between the control and septic groups in these parameters.

5.3. Development of a species-specific pig SOFA scoring system (pSOFA)

The experimental design of our second model was almost the same as before, but we focused on assessing the degree of organ damage by using a human-like SOFA scoring system. Here we propose the use of modified human SOFA-like 3- and 5-domain forms of pSOFA to quantify organ damage comprehensively. The dynamics of the development of organ dysfunction in experimental sepsis may be related to changes in the porcine SOFA score, which may contribute to the human clinical translation of preclinical models. We thus propose that the standardization

of large animal studies with the pSOFA scoring systems will make it possible to compare different models. In our Study 2 setup, we have been able to divide our experimental animals into subgroups according to the severity of the process. An analysis and comparison of our results confirmed the importance of the pig SOFA score system for severity classification. Our previous data also showed the characteristic parameters of septic status, but the separation of sepsis from septic shock significantly nuanced the results, thus reducing the gap between preclinical and clinical outcomes.

5.4 Correlation between degree of microbiological insult and severity of sepsis/ organ damage

A retrospective microbiological analysis was carried out for further investigation in Study 2. Here we also confirmed that the standardized, autologous inoculum resulted in a predominantly E. coli-characterized bacteremia, similarly to many clinical and preclinical observations (Park et al., 2019; Vincent JL et al., 2006). We also examined the relationship between the degree of microbiological invasion and the host response leading to organ dysfunctions characterized by 3D- or 5D-pSOFA scoring. The initial microbial concentration of the inducer inoculum was moderately associated with the severity of organ dysfunction, while the concentration of microbes in the blood showed a much stronger correlation. It is important to note that individual microbiological diversity can contribute to the severity statuses observed in our experimental animals, which may complicate standardization. Therefore, frequent preliminary microflora testing may also facilitate the standardization of an autologous fecal inoculum-induced sepsis model.

5.5. Evaluating the dynamics of the inflammatory markers

The dynamics of all the inflammatory mediators under examination showed significant differences between the sepsis and septic shock groups. Therefore, early (hour 6) detection of plasma TNF-α and a somewhat late elevation of bET and HMGB1 levels may indicate the probability of septic shock-linked organ dysfunction quantified by 3D- and 5D-pSOFA scores as well. Hence, we suggest that these biomarkers may also be of potentially predictive importance in experimental sepsis. In sepsis, macrophages and neutrophils produce reactive oxygen species (ROS) in response to infection. NO and ROS together create a feedback mechanism that boosts the production of both and exacerbates the inflammatory response, thereby increasing plasma NO_x levels (Waltz et al., 2015). At hour 16 in our experiments, the difference between the septic groups was already clearly visible, with higher NO_x levels in the septic shock group supporting this positive feedback. Blood lactate level is an important prognostic marker of sepsis. Higher lactate levels are associated with a worse clinical outcome and greater mortality risk (Rello et al., 2017). In sepsis, higher tissue oxygen consumption and

lower oxygen delivery due to concomitant disturbance of the microcirculation lead to a discrepancy in tissue oxygenation, with increased lactate production due to tissue hypoxia (Lee et al., 2021). In Study 2, growth in blood lactate levels was observed in sepsis in both septic groups as early as 6 hours after induction, which remained persistently high in the septic shock group, indicating persistent tissue hypoxia. This indirectly indicates a reduction of oxygen delivery capacity. In our experimental model, sepsis-associated biomarkers change in the same way as observed in human sepsis. This makes the model suitable both for studying these markers and for developing new therapeutic targets.

5.6. Evaluating oxygen dynamics and microcirculation

The oxygen dynamic parameters in Study 2 differed less between the animals in the sepsis and septic groups within the observation period. The lower SVRI, higher DO_2 and VO_2, and markedly higher ExO_2 demonstrated a compensated hyperdynamic state in this model of progressive sepsis. In Study 2, both septic groups showed a significantly deteriorated microvascular perfusion (reduced PPV values) in the sublingual mucosa. At the end of the monitoring period (hour 24 of the experiments), an improved capillary perfusion rate was observed, which could be explained by the introduction of the organ protective treatments applied (fluid and inotropic therapy, and urinary diuresis).

6. SUMMARY OF NEW FINDINGS

1. We designed and described a clinically relevant large animal model of intra-abdominal sepsis, characterized by complex macro- and microcirculatory changes and human-like changes in inflammatory biomarkers and signs of organ dysfunction. The methodology combines the advantages of conscious and anesthetized studies, and mimics human sepsis and multi-organ failure very closely.
2. The host responses were quantified with modified human SOFA-like 3D and 5D pig SOFA scoring systems. 3D-pSOFA scoring is suitable for evaluating cardiovascular, pulmonary, and renal dysfunctions online without laboratory biochemical testing, while using the 5D-pSOFA score improves reproducibility and provides an alternative endpoint in lieu of mortality.
3. The standardization of large animal studies with the pSOFA scoring systems will make different models comparable, thus reducing the gap between preclinical and clinical outcomes.
4. Microbiological analysis showed that the microbial concentration of the inoculum was closely related to the severity of sepsis and organ damage.

5. Our preclinical model has enabled us to demonstrate the predictive value of inflammation-associated tissue hypoxia or necrosis-induced elevation of plasma biomarkers in identifying late-onset organ damage.

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LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Érces, D., **Zsikai, B.,** Bizánc, L., Sztányi, P., Vida, G., Boros, M., Jiga, L., Ionac, M., Mándi, Y., Kaszaki, J. (2011). An improved model of severe sepsis in pigs. *Timisoara Medical Journal, 61* (3–4), 135–140. SJR Q4;

II. **Zsikai, B.,** Bizánc, L., Sztányi, P., Vida, G., Nagy, E., Jiga, L., Ionac, M., Érces, D., Boros, M., & Kaszaki, J. (2012). Klinikailag releváns sepsismodell törpesertésen [Clinically relevant sepsis model in minipigs]. *Magyar Sebészet, 65*(4), 198–204. doi: 10.1556/maseb.65.2012.4.5

III. Rutai, A*., **Zsikai, B***., Tallósy, S. P., Érces, D., Bizánc, L., Juhász, L., Poles, M. Z., Sóki, J., Baaity, Z., Fejes, R., Varga, G., Földesi, I., Burián, K., Szabó, A., Boros, M., Kaszaki, J. (2022). A Porcine Sepsis Model with Numerical Scoring for Early Prediction of Severity. *Frontiers in Medicine, 9*, 867796. doi: 10.3389/fmed.2022.867796; SJR Q1; IF 3.9; *These authors have contributed equally to this work and share first authorship.