TEMPORAL CHANGES OF THE MICROCIRCULATORY-MITOCHONDRIAL FUNCTIONS IN EXPERIMENTAL RODENT SEPSIS

Ph.D. Thesis

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List of publications

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2. László Juhász, Attila Rutai, Roland Fejes, Szabolcs Péter Tallósy, Marietta Zita Poles, Andrea Szabó, Imre Szatmári, Ferenc Fülöp, László Vécsei, Mihály Boros, József Kaszaki. **Divergent Effects of the N-Methyl-D-Aspartate Receptor Antagonist Kynurenic Acid and the Synthetic Analog SZR-72 on Microcirculatory and Mitochondrial Dysfunction in Experimental Sepsis.** *Frontiers in Medicine,* 2020; 7:566582. **IF: 5.09; Q1**
3. Attila Rutai, Bettina Zsikai, Szabolcs Péter Tallósy, Dániel Érces, Lajos Bizánc, László Juhász, Marietta Zita Poles, József Sóki, Zain Baaity, Roland Fejes, Gabriella Varga, Imre Földesi, Katalin Burián, Andrea Szabó, Mihály Boros, József Kaszaki. **A Porcine Sepsis Model with Numerical Scoring for Early Prediction of Severity.** *Frontiers in Medicine,* 2022; 9:867796. **IF: 5.09; Q1**
4. Roland Fejes, Tamás Szűcsborus, András Czombos, Csaba Góg, Zoltán Ruzsa. **Managing Patients with Overlapping High Risk for Bleeding and Thromboembolic Events.** *Cureus Journal of Medical Science,* 2024; 16(2):e53557. **IF: 1.2**
5. Fejes Roland,* Kovács-Huber Róbert,* Góg Csaba, Kádár Csilla. **Intenzív Inzulinkezelés Korai Deeszkalálásának Lehetőségei [Options for Early De-escalation of Intensive Insulin Treatment].** *Magyar Belorvosi Archívum,* 2024; 77: 45–48. (*equal contribution) **IF: 0**

1. INTRODUCTION

The latest Sepsis-3 definition characterizes sepsis as a life-threatening multi-organ failure (MOF) caused by a dysregulated host response to infection. Diagnosis relies heavily on the Sequential Organ Failure Assessment score, with an increase of ≥ 2 points. Due to the complex pathophysiology, the clinical management of sepsis is still challenging. The immune response in sepsis is triggered by either pathogen- or damage-associated molecular patterns, which activate immune cells and initiate a cascade of proinflammatory responses. Traditionally, sepsis has been depicted as a biphasic model, with an early hyperinflammatory cytokine storm followed by immune paralysis.

Experimental models are crucial for developing potential sepsis treatments, but their findings often cannot be applied to clinical practice due to challenges like standardizing bacterial load and identifying microbiome composition. The most recent Minimum Quality Threshold in Preclinical Sepsis Studies criteria outline a recommended scheme for rodent sepsis and highlight the most important aspects of model development. Rodent models, such as intraabdominal administration of fecal matter, are commonly used to mimic human peritonitis-related sepsis. However, variability in microbial communities and the role of dominant bacterial strains in determining sepsis severity remain poorly understood.

Although sepsis significantly impacts the whole cardiopulmonary system, microcirculatory damage serves as a better indicator for MOF and mortality than traditional macrohemodynamic parameters. Microcirculatory dysfunction plays a key role in the progression of septic MOF, with endothelial cell damage and impaired blood flow contribution. Additionally, microcirculatory functions are strongly related to the subcellular oxygen consumption, mostly represented by mitochondrial respiration. Mitochondrial functional dysfunction and structural damage contribute to the development of MOF with the breakdown of energy-producing mechanisms. The concept of microcirculatory and mitochondrial distress syndrome has been proposed to capture the intertwined nature of these processes, with recent research focusing on supporting microcirculatory oxygen delivery to maintain mitochondrial function. In this outline, mitochondria together with their supplier microcirculatory network are both major drivers of MOF. However, it remains an open question whether these changes are causally related and, if so, how this bidirectional connection develops in time. It is challenging to demonstrate causal links between the two arms of MMD even in standardized experimental conditions due to the web of connections and cross-reactions. Of note, microcirculatory and mitochondrial changes have not been investigated simultaneously either in preclinical or in human sepsis in the pre- and post-MOF periods.

2. MAIN GOALS

1. Given the importance of a prolonged duration of clinical sepsis, we aimed to investigate the appropriate temporal characteristics of a standardized fecal inoculum-induced sepsis (6–72h) in terms of animal well-being and the development of organ dysfunction.
2. As the severity of sepsis may vary between individuals (including rats), we hypothesized that the bacterial composition within the fecal mass that initiates the insults could be a highly important confounding factor and a decisive descriptor of the course of a septic scenario. Therefore, we retrospectively investigated how the microbiological parameters of the sepsis-inducing fecal inoculum influences the severity of intraabdominal sepsis.
3. Our third objective was to determine how the composition and germ count of the bacterial population changes in the abdominal cavity as a function of time and how these factors influence sepsis severity.
4. Since the recent literature suggests a link between sepsis-induced microcirculatory and mitochondrial dysfunction and clinical outcomes, we aimed to investigate the effects of these pathophysiological components on sepsis severity. Additionally, we aimed to simultaneously monitor the splanchnic microvascular status and concurrent mitochondrial function and to characterize their relationship and time dependency in our intraabdominal sepsis model.

3. MATERIALS AND METHODS

This thesis is based on two different studies. In a preliminary study, we examined the effects of filtration and incubation on the microbial characteristics of the inoculum. Based on these results, we were able to use an inoculum with a narrowed microbial concentration range to describe the temporal change of severity. In Study 1, our primary objective was to optimize our polymicrobial sepsis model in which the course of MOF associated with the septic process can be characterized. Since the severity of sepsis was more profound in certain experimental animals, we retrospectively investigated how qualitative characteristics of the inoculum influenced the outcome of sepsis. In Study 2, we focused on the correlations between parameters describing microcirculatory and mitochondrial function and how these correlate with sepsis severity or predict the outcome of sepsis.

3.1. Ethical permissions and animals used in the experiments

The study was approved by the University of Szeged Animal Welfare Committee and the National Scientific and Ethical Committee, the national competent authority in Hungary (ETT-TUKEB; license number: V/175/2018). All institutional and national guidelines for the care and use of laboratory animals were followed.

In the experiments, we used male Sprague Dawley rats that were previously housed in plastic cages in a temperature-controlled room (21–23°C) with a daily 12/12h light and dark cycle. They had unrestricted access to standard rodent chow and water.

3.2. Aims, protocol, and main results of the preliminary study

We aimed to induce peritoneal sepsis with injections of standardized bacterial counts within a defined range but without limiting the variability in the microbiome composition. Therefore, we ran a preliminary study aiming **(1)** to clarify the effects of filtration on the inocula, **(2)** to determine the optimal germ count in the inducing inocula confirmed with a prognosis analysis, and **(3)** to optimize the duration of the incubation.

The general well-being of the animals was assessed using a 0–9-point rat-specific sickness scoring (RSS) system, where a cumulative value at 6 was considered a humane endpoint for euthanasia. There was no significantly elevated RSS value and mortality below 1.02×10^6 colony-forming units (CFUs), and no significant changes occurred in the sickness score for 24h. However, microbial concentration above 5.6×10^6 CFUs resulted in a mortality rate > 90% in the first 12–16h. Therefore, bacterial content between 1.02×10^6 and 5.6×10^6 CFUs was administered to investigate sepsis-associated organ dysfunctions and changes in Study 1 and Study 2.

In addition, the influence of filtration was also examined in the context of mortality. We found that mortality was independent of the germ count when induction was performed without filtration. However, a higher germ count meant higher mortality with filtration due to the reduced random variability of fecal flocculants providing an adhesion surface for living microorganisms. The qualitative microbiological analysis (see method in Section 3.3) demonstrated a significant decrease in the number of bacterial strains after only 10h of incubation at 37°C because of the log phase of microbial competition for population growth.

3.3. Microbial characterization of the fecal inoculum

For microbiological analysis, 0.1 mL samples of the inocula were used to determine CFU and identify the bacterial strains. CFU was determined using the standard pour-plate count method. Species-selective media and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS; Bruker Daltonics, Germany) were employed to analyze the bacterial composition of the inoculum. The results of the bacterial composition were typically available 48h after the completion of the inoculum preparation procedure.

3.4. Experimental protocol in Study 1

The animals (380 ± 30 g) were randomly assigned to sham-operated ($n_{\sum} = 49$) and septic groups ($n_{\sum} = 51$), which were randomly further divided into four independent groups each (sham-operated: $n_{12h} = 13$, $n_{24h} = 12$, $n_{48h} = 12$, $n_{72h} = 12$; septic: $n_{12h} = 13$, $n_{24h} = 13$, $n_{48h} = 13$, and $n_{72h} = 12$)

according to a termination timeline. Based on the data obtained in the preliminary study, the filtered inoculum was injected intraperitoneally (ip) using a 21G needle at a volume of 5 mL kg^{-1}. The rats in the sham-operated groups received saline in the same volume. The general condition of the animals was evaluated at 6h after the ip injections and every 12h thereafter using the RSS scoring system. At time points in the RSS assessment, the animals received 10 mL kg^{-1} crystalloid solution (Ringerfundin, B. Braun, Hungary) subcutaneously (sc) to avoid dehydration and 15 µg kg^{-1} buprenorphine sc (Bupaq, Merck, USA) sc as analgesia. At the end of progression in each group, the animals were anesthetized and hemodynamic monitoring, blood and tissue sampling, and ascites sampling for microbiological measurements were performed. At the end of the protocol, the experimental animals were sacrificed under deep anesthesia with an overdose of ketamine (120 mg kg^{-1}).

3.5. Experimental protocol in Study 2

The animals were randomly assigned to sham-operated ($n_{\sum} = 40$) and septic groups ($n_{\sum} = 40$), which were randomly further divided into five independent subgroups each ($n_{12h} = 8$, $n_{16h} = 8$, $n_{20h} = 8$, $n_{24h} = 8$, and $n_{28h} = 8$) according to the different time points of sepsis progression. Following 6, 12, 16, 20, or 24h of sepsis progression, the animals received fluid therapy and analgesia, and RSS was evaluated. Subsequently, the animals were anesthetized, and invasive hemodynamic monitoring was initiated (see Section 3.6). Thereafter, a median laparotomy was performed to observe the ileal microcirculation with a serosal orientation with the Incident Dark Field (IDF) imaging technique (see Section 3.8). Immediately after microcirculatory measurements, a liver tissue biopsy was taken to assess mitochondrial respiratory functions with high-resolution fluorespirometry (see Section 3.9). Afterward, blood samples were collected from the inferior vena cava to measure inflammatory and organ failure markers.

3.6. Anesthesia, surgical preparation, and invasive cardiopulmonary monitoring

In both studies, the animals were subjected to anesthesia by ip injection of a mixture of ketamine (45.7 mg kg^{-1}) and xylazine (9.1 mg kg^{-1}). Thereafter, the animals were positioned supine on a heating pad set to 37°C. Tracheostomy was performed, with the right jugular vein cannulated for crystalloid infusion and continuous anesthesia. The left carotid artery was cannulated to monitor mean arterial pressure (MAP) and a thermistor-tip catheter was inserted into the contralateral carotid artery to measure cardiac output (CO) (Cardiosys 1.4, Experimetria Ltd., Budapest, Hungary). Hemodynamic parameters were recorded for the duration of the 60-min observation period. At the 60^{th} min of the monitoring period, arterial blood samples were collected for blood gas analysis (Cobas b123; Roche Ltd., Basel, Switzerland). Oxygen delivery, consumption, and extraction (DO_2, VO_2, and ExO_2, respectively) were calculated using standard equations. Lung function was assessed

by determining the ratio of arterial partial pressure of oxygen to the fraction of inspired oxygen (PaO_2 FiO_2^{-1}, where $FiO_2 = 0.21$). Following the 60-min hemodynamic monitoring, a median laparotomy was conducted to observe the microcirculation of the ileal serosa (see Section 3.8). After microcirculatory measurements, a liver tissue sample was taken to assess mitochondrial respiration (see Section 3.9).

3.7. Examination of serum markers; assessment of ROFA score

Whole blood lactate levels were measured from venous blood (Accutrend Plus Kit; Roche Diagnostics Ltd., Rotkreuz, Switzerland). Blood samples were collected from the inferior vena cava in EDTA-coated tubes, centrifuged (1.200g at 4°C for 10 min), and stored at −70°C. Plasma interleukin-6 (IL-6) level and endothelin-1 (ET-1) levels were determined according to standard enzyme-linked immunosorbent assay kit protocols (Cusabio Biotechnology Ltd., Wuhan, China). Kidney function was characterized by plasma urea level. Liver function was assessed by plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Roche/Hitachi 917 analyzer; F. Hoffmann–La Roche AG, Switzerland). A rat-specific organ failure assessment (ROFA) scoring system was used to describe MOF. Sepsis was defined as a cumulative ROFA score over 2.

3.8. Examination of ileal microcirculation with Incident Dark Field imaging

Ileal microcirculation was visualized with a serosal orientation using the IDF imaging technique (CytoCam Video Microscope System; Braedius Medical, Huizen, the Netherlands). After the median laparotomy, an ileal loop was removed from the abdomen intact and placed on a special holder. The CytoCam device consists of LEDs emitting guided light with a wavelength of 530 nm, which is absorbed by hemoglobin-containing particles (red blood cells). Images from an ileum segment were recorded in six, 50-frame, high-quality video clips. The records were analyzed with an offline software-assisted system. The proportion of perfused vessels (PPV) was defined as the ratio of the perfused vessel lengths to total vessel lengths. To evaluate microcirculatory heterogeneity, after a semiquantitative analysis, individual vessels were distinguished between (0) absent, (1) intermittent, (2) sluggish, and (3) continuous flow. A value was assigned to each vessel. and the overall score for each record was the average of the individual values, indicating the microvascular flow index (MFI). The heterogeneity index (HI) was defined as the difference between the highest MFI and the lowest MFI divided by the average MFI of the record.

3.9. Assessment of hepatic mitochondrial functions with high-resolution respirometry

In both studies, mitochondrial oxygen consumption ($mtVO_2$) was measured in liver homogenates using high-resolution fluorespirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). In brief, liver samples obtained from the left lateral lobe were homogenized,

and then respirometry was performed in a MiR05 respiration medium at a constant temperature with continuous stirring (37°C, 750 rpm). After stabilization of VO_2, rotenone was added (1) to inhibit Complex I activity and (2) to prevent accumulation of oxaloacetate (a known endogenous inhibitor of Complex II). $FADH_2$-supported LEAK respiration and maximal capacity of oxidative phosphorylation (OxPhos) were determined in the presence of exogenous succinate and adenosine diphosphate (ADP). Following stimulation of OxPhos, the integrity of the mitochondrial outer membrane (mtOM) was tested with the addition of exogenous cytochrome c, and the increase in $mtVO_2$ was expressed as a percentage of $mtVO_2$ as compared to $mtVO_2$ in OxPhos (CytC%). Complex V (or ATP synthase) was inhibited with oligomycin to assess leak respiration in a non-phosphorylating state ($LEAK_{Omy}$) and to calculate respiratory control ratio (RCR), an index of the coupling of mitochondrial respiration to OxPhos ($Oxphos/LEAK_{Omy}$). DatLab 7.3.0.3. software (Oroboros Instruments, Innsbruck, Austria) was used for online display and respirometry data acquisition and analysis.

3.10. Statistical analysis

Data were evaluated with the SigmaStat 13 software package (Systat Software, San Jose, CA). The survival rate was analyzed and plotted using the Kaplan–Meier method. The Mann–Whitney or Kruskal–Wallis test was used with Dunn's post-hoc test for discrete variables, while a two-way ANOVA was employed for continuous variables followed by the Holm–Sidak post-hoc test. Data were displayed as median values and interquartile ranges between the 75^{th} and 25^{th} percentiles, with $P < 0.05$ being considered significant. The receiver operating characteristic (ROC) curve analysis was used to represent the diagnostic accuracy of the different parameters determined by the area under the curve (AUC) data (GraphPad Prism 8.0). Pearson's method or Spearman's methods were employed to analyze the linear correlation, correlation coefficient (r), regression lines, and 95% confidence intervals.

4. RESULTS

4.1. Results of Study 1

4.1.1. Animal well-being; mortality

Within 24h, the condition of some septic animals deteriorated significantly, RSS reached a critical value of 6, and, therefore, n = 3, n = 4, and n = 3 animals were euthanized in Groups 24h, 48h, and 72h, respectively. The numbers of euthanized animals are included in the mortality calculations. The general condition of the surviving septic animals did not deteriorate between 48 and 72h.

4.1.2. Global and subcellular oxygen dynamics; inflammatory markers

There was no significant difference in ExO_2 between the sham-operated and septic animals at 12h, but the values showed a significant reduction after 24h of sepsis. Less diminished ExO_2 values were detected in the septic groups. The Complex II-linked OxPhos also showed deterioration in the 24h septic animals only. Elevated levels of plasma IL-6 were observed 12h after induction; plasma ET-1 concentration increased significantly at 24h of sepsis.

4.1.3. Changes in ROFA score

The ROFA score showed significant increases at 24 and 48h. Deteriorations in the 24h values were attributable to all parameters examined, whereas similar changes were also present for most parameters at 48h, except for the not significantly different whole blood lactate and plasma ALT values. ROFA scores returned close to the values for the sham-operated animals after 72h.

4.1.4. Microbial background of the fecal inoculum and the ascites

In addition to determining CFU, analysis of the bacterial pattern was performed from both the sepsis-inducing fecal inocula and the ascites. This retrospective microbial analysis revealed marked qualitative differences in bacterial composition and monomicrobial/polymicrobial patterns in the composition of the inocula. E. coli was present in 20% of inocula ($n_{\Sigma} = 10$) and all early (24h) sepsis mortality was attributable to injection with E. coli monomicrobial cultures. In the sepsis-inducing inocula, three bacterial phyla, including 18, mostly Gram-negative genera, could be detected. Most taxa belonged to Proteobacteria (49%; e.g., E. coli), and the rest were distributed amongst Firmicutes (38%; e.g., Lactobacilli) and Actinobacteria (13%; e.g., *Propionibacterium acnes*). The most frequent strains in the inducer inoculum were *E. coli* (in 100% of the samples). In the ascites, bacterium concentration decreased by one order of magnitude, which was accompanied by reduced diversity of bacterial strains. New species also reached the detection level. After 72h of sepsis, only E. coli (in 100% of the samples) and *Lactobacillus murinus* (in 17% of the samples) were identified in the ascites.

4.1.5. Association between the inducing bacterial dose and the severity of organ failure

The initial CFU and ROFA scores showed a moderate relationship at 12h. However, a significant correlation between these parameters was observed at 24h of fecal peritonitis. No connection between the amount of injected CFU and the ROFA score was evidenced at 48 and 72h.

4.1.6. ROFA score components at 24 hours after mono- and polymicrobial inocula

Since marked differences in the diversity of bacterial strains were revealed in the inoculum, we ran a retrospective subgroup analysis to compare the parameters of ROFA scores for animals that were found to be challenged with polymicrobial inoculum (in the 24h sepsis group; n = 10) and with E. coli monomicrobial content in the 24, 48, and 72h sepsis groups (n = 3, 4, and 3 animals,

respectively). We found significantly higher ROFA score values in the animals injected with E. coli monomicrobial inoculum compared to the other cohort. Bearing in mind the negligible quantitative differences in inocula but the differences in their microbiological diversity, we also retrospectively assessed how simultaneous consideration of these features influenced organ dysfunction at 24h of sepsis. The relationship between the CFU values and ROFA scores showed a similar correlation in both the E. coli monomicrobial and the polymicrobial subgroups, but the slope of the regression line of the monomicrobial subgroup was greater than that of the polymicrobial subgroup. Furthermore, the ROFA score also displayed a correlation with ascites CFU values. A significant non-linear relationship was identified between the ascites CFU and the ROFA score in the polymicrobial or monomicrobial subgroups during the 12–72h sepsis period with a shifting of the bacterial composition towards E. coli dominance over time.

4.2. Results of Study 2

4.2.1. Animal well-being and mortality

There were no significant changes in the RSS score in the sham-operated group. The septic groups between 20 and 24h elevated significantly not only compared to the 12h septic group but also compared to the 20 and 24h sham-operated groups. Three animals were euthanized due to a critically elevated RSS score.

4.2.2. Changes in inflammatory and multi-organ failure markers

IL-6 significantly increased as compared to the sham-operated group, peaking at 16h, after which a decrease started reaching the level of the sham-operated group after 24h. The ROFA parameters were significantly higher in the septic groups compared to the sham-operated animals during the whole experimental period, with the score reaching the maximum at 20h.

4.2.3. Changes in macrohemodynamics and oxygen dynamics

In the sham-operated group, there were no significant hemodynamic changes over time. Sepsis was characterized by significant hypotension between 16 and 24h compared to the sham-operated animals. An increasing trend was observed in CO data until 16h, and then CO dropped significantly below the level of the sham-operated animals at 20h. In the 16–24h interval, the CO values differed significantly from the values for the 12h group, albeit in a different way. DO_2 showed a progressive decline in the septic group, reaching its lowest value at 20h, thereafter exceeding the levels for the sham-operated animals at 28h. There was no significant change in VO_2 except at 16h. ExO_2 rose until 20h, when the trend reversed and did not alter from the values for the sham-operated groups. In the 16–24h interval, the ExO_2 values differed significantly from the values for the 12h septic group.

4.2.4. Changes in microhemodynamics

PPV and MFI were significantly higher compared to the sham-operated group between the 16 and 28h interval. HI was significantly higher in the 16–28h interval with a decreasing trend in the septic group but still significantly higher compared to the sham-operated group at 28h.

4.2.5. Changes in liver mitochondrial functions

The OxPhos and $LEAK_{Omy}$ values for the septic animals significantly decreased at 20h compared to the sham-operated group. CytC%, which characterizes mtOM damage, showed a strong increasing trend with a maximum at 20h and was significantly different from the sham-operated groups between 16 and 28h. RCR did not differ significantly in the septic animals compared to the sham-operated ones during the whole experimental period.

4.2.6. Receiver operating characteristic (ROC) curve analyses

The diagnostic power of the parameters was determined with the calculated AUC values from the ROC classification analysis. Among the parameters of the ROFA score, only whole blood lactate level showed relevant predictive value. Among the mitochondrial functions, CytC% proved to be a strong predictor. Among the microcirculatory parameters, PPV showed the strongest predictive value, and both MFI and HI had a particularly high predictive value.

4.2.7. Correlation between MMD parameters and the ROFA score

There was no significant correlation in the sham-operated groups between the microcirculatory-mitochondrial variables and the ROFA score. There was a negative, significant correlation between PPV and the ROFA score and a positive, significant correlation between HI and the ROFA score in the sepsis group. There were no relations between the mitochondrial parameters and the ROFA score. There was no significant correlation in the sham-operated groups between the mitochondrial and microcirculatory variables. In the sepsis group, there was no significant correlation between the microcirculation and OxPhos variables. Furthermore, there was a negative, significant correlation between PPV and CytC%. However, HI and CytC% also showed a moderate, positive relationship.

5. DISCUSSION

5.1. The impact of the microbial composition of inocula on sepsis progression

Several preclinical animal models of sepsis with various advantages and disadvantages have already been presented to the scientific community. Fecal inductions with standardized CFU concentration ranges are considered a suitable methodology. In our hands, 1.02×10^6–5.6×10^6 CFUs correlated well with the onset time of severe reactions 24h later. This CFU range resulted in approx. 20% summed mortality, broadly reproducing the mortality rate of human intraabdominal sepsis. Approximately 60% of human septic cases are caused by bacterial

infections, and Gram-positive species are detected in the hemoculture in 46% of cases. Monomicrobial origin is rare in human sepsis. The literature agrees that E. coli is one of the most common pathogens, and, although the respiratory tract is the most frequent source of infection, an abdominal origin also makes up a significant percentage. Still, the identification of endogenous or exogenous microbial sources is usually lacking in the design of animal model strategies. E. coli showed the highest frequency among the sepsis-causing bacteria we isolated (followed by Klebsiella, Pseudomonas, and Acinetobacter), a finding which is consistent with clinical experience. However, sepsis with a Gram-positive source in rodents is relatively rare in contrast to the frequent occurrence of *Staphylococcus aureus* in human sepsis. Here we have demonstrated the paramount importance of identifying inducer strains because approx. 20% of the fecal solutions with a presumed polymicrobial habitat proved to be monomicrobial (with only E. coli present according to our study). According to clinical studies, the progression mainly depends on the pathogen and on the clinicopathologic characteristics of the patient population under examination. In their meta-analysis, Karakonstantis et al. concluded that polymicrobial sepsis has a lower mortality rate; however, the study had a high risk of bias, and we still have weak clinical evidence in this matter.

5.2. Temporal changes in the composition of bacterial content during sepsis progression

It should be underlined that a shift from polymicrobial to monomicrobial content had begun during the preparation of the uniform and mixed stool samples and that the transition was then tracked within the body of the animals. Another distinctive feature of the process was the Lactobacillus and Bifidobacteria cultures detected in the ascites perhaps as a compensatory sign of the host's bacterial defense mechanism. We followed the dynamics of the transition of bacterial strains in the surviving animals; the concentration and diversity of the polymicrobial cultures in the ascites decreased after 24h, and the most frequent strain was again E. coli. However, new strains (e.g., *Neissera subflavia*) were also detected over time during the secondary peritonitis. Finally, the majority of the strains disappeared from the ascites, and only *E. coli* and *Lactobacillus murinus* were detected with reduced concentrations in the 72h samples. The emergence of new species also raises the possibility of interaction between strains. Among these species, Lactobacilli are classified as probiotics and have special significance because they exert beneficial effects on the human body. Their presence is linked to the preservation of gut microbiota homeostasis and the initiation of anti-inflammatory processes, believed to be a result of the compounds they produce. The exact processes underlying the higher survival rate observed following polymicrobial induction are not known, but bacteria-bacteria interaction may play an important role. To our knowledge, our study was the first to demonstrate the

dominant appearance of E. coli monomicrobial cultures in association with early mortality in experimental sepsis. A natural selection that is amplified under favorable conditions is the most likely explanation for the process leading to microbial fitness and the “survival of the meanest” phenomenon.

5.3. The possible contribution of microcirculatory and mitochondrial changes to the severity of organ dysfunction in experimental sepsis

In this set-up, the divergent dynamics of sepsis-induced microcirculatory and mitochondrial responses were discerned with the presence of the whole septic macrohemodynamic pattern with compensatory hyperdynamic, decompensated hypodynamic, and final recovery stages, thus providing insight into the progression of sepsis without resuscitation. The joint feature of microcirculatory-mitochondrial impairment, or MMD, has already been identified as a significant element in the pathogenesis of human MOF, but alterations to intestinal microcirculation and hepatic mitochondrial functions to date have not been studied together in MOF associated with intraabdominal sepsis. Our study partially confirms the literature, showing an early proportionate decrease of perfused microvessels and increased perfusion heterogeneity with matching changes in ROFA scores. Nevertheless, the initial microcirculatory failure was improved and then restored in the later phase. Importantly, the hepatic mitochondrial function deteriorated much later with a rapid recovery of oxidation-linked parameters and a permanently sufficient coupling of the respiratory chain, but CytC% was elevated significantly, suggesting mitochondrial outer membrane (mtOM) damage. Injury to mtOM leads to the partial loss of intramitochondrial CytC and decreased mitochondrial VO_2. We have used an established respirometry method to determine mtOM integrity with the stimulation of mitochondrial respiration following CytC replacement from an outer source. CytC% had a high diagnostic power during the whole course of the 28h experimental period, thus suggesting that it may be a suitable biomarker for the onset of severe sepsis. However, despite this correlation, mtOM damage or OxPhos changes did not influence the ROFA score. It has to be highlighted that the markedly rapid restoration of OxPhos suggests an immense reserve capacity of hepatic mitochondrial respiration to compensate for the loss of membrane integrity. Of note, the correlation between ROFA scores and microcirculatory parameters indicates that the onset of MOF is mainly influenced by microvascular factors, and the correlation between microcirculatory variables and mtOM damage without the involvement of transient loss of oxidative capacities further confirms the presence of strong mitochondrial coping mechanisms.

The pathogenesis of MMD is complex and still only partially known, but it seems that different mitochondrial functionalities may be present in adjacent areas with microvascular flow heterogeneity. The non-uniformity may also result from the heterogeneous ultrastructural,

biophysical, and electrochemical characteristics of liver mitochondria, which may lead to potentially decaying and surviving subpopulations in response to pathologies and post-translational processes. Heterogeneity may also arise from the mitochondrial life cycle itself, including biogenesis, motility, fusion and fission, and the clearance of damaged mitochondria. Thus, we hypothesize that increased CytC efflux – hypothetically expected from the increased membrane permeability – preceded the complete clearance of degraded mitochondria with mtOM damage and that a structurally and metabolically more resilient and compensatory subpopulation was able to maintain undisturbed ATP synthesis. These mitochondria may be maintainers or restorers of microcirculation and tissue integrity in the later period where sepsis is resolved.

In this study design, the ROFA score was used to monitor MOF progression similarly to the human clinical routine, and the diagnostic value of microcirculatory parameters could be established within this scheme. As with human practice, the serum lactate level was also significantly elevated and proved to be a strong predictor of severity, supporting the diagnostic importance of hypoxia markers. However, the low serum level of renal and hepatic dysfunction markers points to the direction of "septic stunning," suggesting that the organs were primed with "hypoxic hibernation" for the expected insult.

Our experimental protocol provided an opportunity to address the chicken or egg causality dilemma of MMD in sepsis. Microcirculation is a significantly early predictor of MOF as compared to mitochondrial respiration. We have used particular parameters to demonstrate that detectable microcirculatory or mitochondrial functional deteriorations occur at different time points depending on the course of sepsis and the compensatory mechanisms and that inappropriate monitoring times may lead to erroneous conclusions. By sequentially tracking the development of MOF, our results shed light on the dynamics of the microvascular oxygen supply, thus providing a better chance for the appropriate timing of diagnostic and therapeutic efforts.

6. SUMMARY AND NEW FINDINGS

1) We have optimized a rat model of fecal inoculation-induced, intraabdominal sepsis in terms of bacterial cell number and incubation time.

2) Our data indicate that the type of inducing and colonizing bacteria in the peritoneal sac significantly influences the outcome. This indicates the need for microbiological analysis when constructing a model of sepsis.

3) The results suggest the presence of competitive intraperitoneal bacterial processes, leading to an increased relative prevalence of certain dominant strains. This may also explain the development of varying sepsis severities.

4) The dynamic analysis of MMD, along with the deterioration of organ functions, demonstrated that these changes occur either in parallel with or as a potential pathophysiological basis for this process. In this context, it should be noted that ileal microvascular perfusion failure occurs earlier and has a higher predictive value for septic MOF than hepatic mitochondrial functional changes. These findings offer valuable translational insights, as they reflect mechanisms that may also be relevant in human sepsis pathophysiology.

5) Hepatic oxidative mitochondrial functions recover relatively rapidly, thus potentially indicating the existence of mitochondrial subpopulations with different responses to tissue hypoxia.

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