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Vagus Nerve Stimulation Attenuates Multiple Organ Dysfunction in Resuscitated Porcine Progressive Sepsis

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Objectives: To investigate the potential benefits of vagus nerve stimulation in a clinically-relevant large animal model of progressive sepsis.

Design: Prospective, controlled, randomized trial.

Setting: University animal research laboratory.

Subjects: Twenty -five domestic pigs were divided into three groups: 1) sepsis group (eight pigs), 2) sepsis + vagus nerve stimulation group (nine pigs), and 3) control sham group (eight pigs).

Interventions: Sepsis was induced by cultivated autologous feces inoculation in anesthetized, mechanically ventilated, and surgically instrumented pigs and followed for 24 hours. Electrical stimulation of the cervical vagus nerve was initiated 6 hours after the induction of peritonitis and maintained throughout the experiment.

Measurements and Main Results: Measurements of hemodynamics, electrocardiography, biochemistry, blood gases, cytokines, and blood cells were collected at baseline Gust before peritonitis induction) and at the end of thein vivo experiment (24 hr after peritonitis induction). Subsequent in vitro analyses addressed cardiac contractility and calcium handling in isolated tissues and myocytes and analyzed mitochondria function by ultrasensitive oxygraphy. Vagus nerve stimulation partially or completely prevented the development of hyperlactatemia, hyperdynamic circulation, cellular myocardial depression, shift in sympathovagal balance toward sympathetic dominance, and cardiac mitochondrial dysfunction, and reduced the number of activated monocytes. Sequential Organ Failure Assessment scores and vasopressor requirements significantly decreased after vagus nerve stimulation.

Conclusions: In a clinically-relevant large animal model of progressive sepsis, vagus nerve stimulation was associated with a number of beneficial effects that resulted in significantly attenuated multiple organ dysfunction and reduced vasopressor and fluid resuscitation requirements. This suggests that vagus nerve stimulation might provide a significant therapeutic potential that warrants further thorough investigation. *(Crit Care Med* 2019; 47:e461- e469)

Key Words: organ dysfunction; pig; sepsis; shock; vagus nerve stimulation

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n an inspirational series of studies, the vagus nerve was identified as an important part of the afferent and effer- ent arms of neural circuits that can regulate the immune response, and the concepts of the cholinergic anti-inflammatory pathway and the inflammatory reflex were established (I-4). According to this concept, the afferent signals induced by endogenous and exogenous products of inflammatories (cytokines, eicosanoid,s pathogen-, and danger-associated

molecular patterns) are transmitted through the vagus nerve to the nucleus tractus solitarii, which relays the signals further to nuclei in the brain stem and hypothalamus (5). The efferent signals travel back via the vagus nerve to the celiac ganglia, in which the adrenergic splenic neurons are activated and transmit signals to the spleen (4, 6). Macrophages of the spleen are then modulated through either 7 subunit nicotinic acetylcholine receptors or 2-adrenergic receptors to down-regulate in ammatory mediator production (7±9).

Extensive experimental work demonstrated that cholinergic/vagus nerve stimulation inhibits cytokine production and activities and improves disease endpoints in a number of experimental rodent models of systemic in ammation and sepsis (10±14).

Although the body of evidence accumulated so far in favor of the cholinergic anti-in ammatory pathway is impressive, to the best of our knowledge, the therapeutic potential in sepsis/endotoxemia was only tested in short-term small animal (mice, rats) models. However, host susceptibility to pathogenic factors and the characteristics of immune, in ammatory, metabolic, and hemodynamic responses in these rodent models do not mimic human stress responses. Consequently, the translation of these experimental ndings to the clinical level is limited (15).

In this study, we addressed this potential shortcoming and examined possible therapeutic effects of vagus nerve stimulation in a clinically relevant porcine model of peritonitis-induced progressive sepsis. This model closely mimics human pathophysiology, with hyperdynamic circulation, low systemic vascular resistance, and multiple organ dysfunction.

MATERIALS AND METHODS

Animal handling was performed in accordance with the European Directive forthe Protectionof Vertebrate Animals Used for Experimental and Other Scienti c Purposes (86/609/EU). The experiments were approved by the Committee for Experiments on Animals of the Charles University Faculty of Medicine in Pilsen and by the Ministry of Education, Youth, and Sports of the Czech Republic (protocol number MSMT-24725/2014±5). All experiments were performed in the Laboratory of Experimental Intensive Care Medicine of the Biomedical Center at the Faculty of Medicine in Pilsen. Twenty- ve domestic pigs of both sexes (18 barrows, seven sows) and of similar weight (44.5 5.7 kg) were randomly divided into three groups: 1) sepsis group (eight pigs), 2) sepsis + vagus nerve stimulation group (nine pigs), and 3) control sham group (eight pigs).

Anesthesia, Instrumentation, and Experimental Protocols

Anesthesia, instrumentation, and experimental protocols were similar to those previously described (16, 17) and are provided in detail in **Supplemental Materials and Methods** (Supplemental Digital Content 1, http://links.lww.com/CCM/E498). In the vagus nerve stimulation group, the cervical portion of the left vagus nerve was exposed and attached to the bipolar stimulation electrode (Harvard Apparatus, Holliston, MA). The stimulation started 6 hours after the induction of peritonitis and continued until the end of the experiment. The vagus nerve was stimulated by rectangular impulses (frequency 2 Hz, amplitude 5 mA, duration 2 ms) using a constant current stimulus isolator with an integrated pulse generator (Isostim A320; WPI, Sarasota, FL).

Measurements and Analysis

The measurements and analyses were similar to those previously described (16, 17) and are provided in the Supplemental Digital Content in detail.

RESULTS

All animals survived during the 24 hours of resuscitated experimental sepsis. Sequential Organ Failure Assessment (SOFA) score values were signi cantly elevated in the septic group (**Fig. 1*A***), although the individual responses to the infectious stimulus were heterogeneous, and two animals did not meet Sepsis-3 criteria for the development of sepsis (**Supplemental Fig. 1**, Supplemental Digital Content 2, http://links.lww.com/CCM/E499; **legend**, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). At the start of the vagus nerve stimulation (6 hr after induction of peritonitis), there were no statistically signi cant differences in any measured variables between sepsis and sepsis with vagus nerve stimulation groups (data not shown). In the vagus nerve stimulation group, sepsis progression was attenuated, as documented by SOFA scores similar to control 24 hours after the induction of peritonitis (Fig. 1*A*). In the organ systems that were included in the SOFA score, bene cial effects of vagus nerve stimulation were found in the respiratory system (Pao_2/Fio_2 of 361.5 mm Hg [85±376 mm Hg] in sepsis vs 447 mm Hg [132±176.5 mm Hg] in sepsis with vagal stimulation; $p < 0.05$) and in the liver (serum aspartate transaminase of 2.6 kat/L [1.4±7.9 kat/L] in sepsis vs 1.6 kat/L [0.5±1.1 kat/L] in sepsis with vagal stimulation; $p < 0.05$). In the kidney, the vagus nerve stimulation did not affect either plasma creatinine levels (122.5 mol/L [125±187 mol/L] in sepsis vs 110 mol/L [36±82 mol/L] in sepsis with vagal stimulation; $p > 0.05$) or the acute kidney injury (AKI) frequency (AKI present in 4/8 pigs with sepsis vs 3/9 pigs with sepsis and vagal stimulation; $p > 0.05$). Platelet counts were similar in both septic groups (120 10^9/L [44±145 10^9/L] in sepsis vs 147 10^9/L [39±73 10^9/L] in sepsis with vagal stimulation; $p > 0.05$). The vagus nerve stimulation completely abolished the sepsis-induced rise in plasma lactate levels (**Fig. 1*B***).

Maintenance of the mean arterial pressure at a level above 65 mm Hg (**Supplemental Table 1**, Supplemental Digital Content 3, http://links.lww.com/CCM/E500), which is required in most septic group animals (in 6/8 pigs with sepsis vs 6/9 pigs with sepsis and vagal stimulation), was achieved through the administration of norepinephrine (**Fig. 1*C***). The total dose of norepinephrine was signi cantly lower in the vagus nerve stimulation group (Fig. 1*C*), although the time to the rst administration of norepinephrine was similar for both septic groups (not shown). Mean infusion rate showed a tendency to decrease in vagus nerve stimulation group,

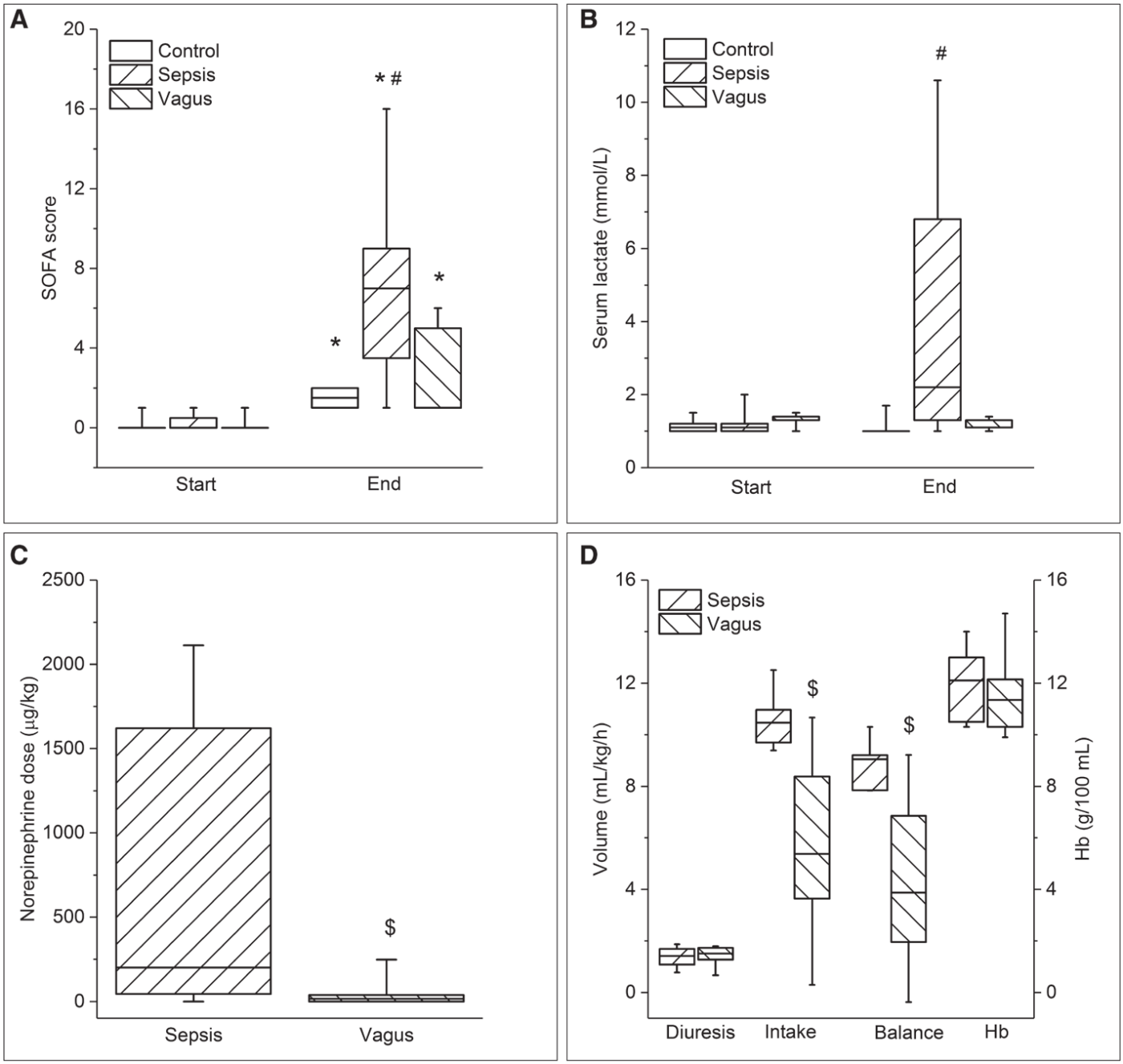

Figure 1. Sequential Organ Failure Assessment (SOFA) score, vasopressor support, and uid resuscitation. **A**, SOFA score in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **B**, Serum lactate levels in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **C**, Total norepinephrine dose in sepsis group (*left box*) and group of sepsis and vagal stimulation (*right box*). **D**, Diuresis, resuscitation uid intake, uid balance (determined as difference of uid intake and of diuresis), and hemoglobin (Hb) concentration in sepsis group (*left box*) and group of sepsis and vagal stimulation (*right box*). **p* < 0.05 versus start; #*p* < 0.05 versus control; $*p* < 0.05 sepsis versus sepsis + vagus stimulation. Data are presented as median with interquartile range (*box*) and total range (*whiskers*).

the difference; however, did not reach statistical signi cance (Supplemental Fig. 1, Supplemental Digital Content 2, http://links.lww.com/CCM/E499; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). Furthermore, vagus nerve stimulation was associated with lower volumes of resuscitation uid, despite the similar diuresis conditions and plasmatic hemoglobin concentrations between the septic groups (**Fig. 1*D***).

Sepsis was associated with hyperdynamic circulation with increased cardiac output (**Fig. 2*A***). Elevated heart rate (**Supplemental Fig. 2**, Supplemental Digital Content 4, http://links.lww.com/CCM/E501; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505) was accompanied with decreased stroke volume (Supplemental Fig. 2, Supplemental Digital Content 4, http://links.lww.com/CCM/E501; legend, Supplemental Digital Content 8,

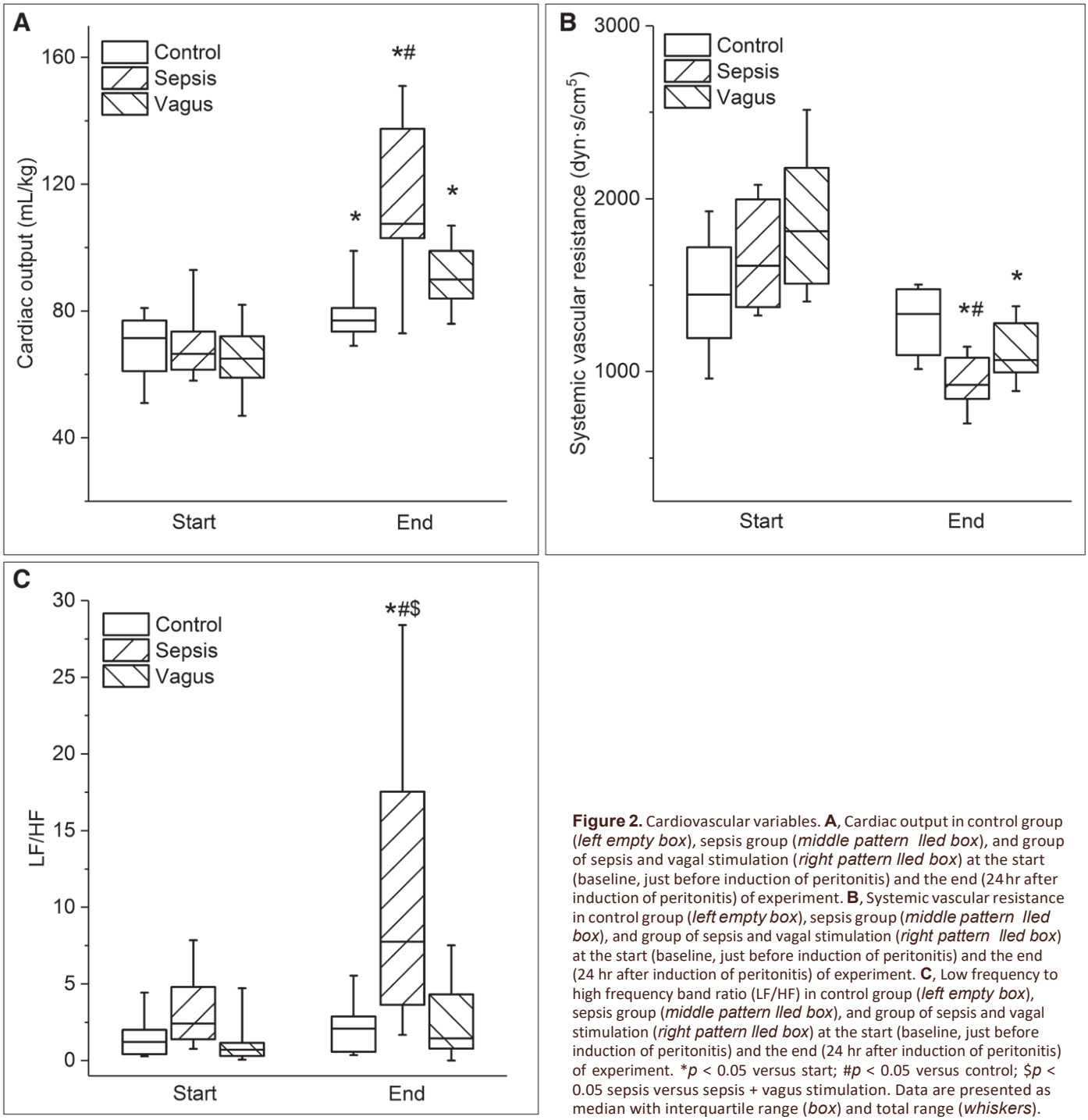

Figure 2. Cardiovascular variables. **A**, Cardiac output in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **B**, Systemic vascular resistance in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **C**, Low frequency to high frequency band ratio (LF/HF) in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **p* < 0.05 versus start; #*p* < 0.05 versus control; $*p* < 0.05 sepsis versus sepsis + vagus stimulation. Data are presented as median with interquartile range (*box*) and total range (*whiskers*).

http://links.lww.com/CCM/E505) and peripheral vasodi- lation (**Fig. 2*B***). Vagus nerve stimulation partially reversed these changes and prevented the cardiac output increase, predominantly by heart rate reduction (Supplemental Fig. 2, Supplemental Digital Content 4, http://links.lww.com/CCM/E500; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). Heart rate variability analysis revealed a signi cant increase in the low frequency to high frequency band ratio in septic animals, which was completely abolished by vagus nerve stimulation (**Fig. 2*C***).

Blood pressures, blood gases, and acid-base balance variables were not improved by vagus nerve stimulation (Supplemental Table 1, Supplemental Digital Content 3, http://links.lww.com/CCM/E500). In all groups, normoglycemia was maintained using 10% glucose infusion (Supplemental Table 1, Supplemental Digital Content 3, http://links.lww.com/CCM/E500). Body temperature was increased in sepsis, and vagus nerve stimulation did not affect it (Supplemental Table 1, Supplemental Digital Content 3, http://links.lww.com/CCM/E500). Arterial 8-Isoprostane levels were similar in all

groups (Supplemental Table 1, Supplemental Digital Content 3, http://links.lww.com/CCM/E500). In multicellular cardiac preparations (trabeculae), the action potential duration at 90% repolarization was not signi cantly in uenced neither by sepsis nor by vagus nerve stimulation (**Supplemental Fig. 3**, Supplemental Digital Content 5, http://links.lww.com/CCM/E502; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). The contraction force was reduced during sepsis, and vagus nerve stimulation completely normalized the contraction force (**Fig. 3*A***). Similarly, in isolated cardiac myocytes, sarcomeric shortening was inhibited by sepsis at low stimulation frequencies and was completely normalized by vagus nerve stimulation (**Fig. 3*B***). In contrast, the calcium transient amplitude, which was also reduced in sepsis, was not affected by vagus nerve stimulation (**Fig. 3*C***). On the other hand, vagus nerve stimulation was associated with elevated baseline intracellular calcium levels (**Fig.3*D***).

Both oxygen delivery and oxygen consumption increased during sepsis, and the vagus nerve stimulation partially suppressed these sepsis-induced changes (**Fig. 4**, ***A*** and ***B***; and **Supplemental Fig. 4**, Supplemental Digital Content 6, http://links.lww.com/CCM/E503; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). The mitochondrial function of the heart was analyzed with ultrasensitive oxygraphy, which revealed a signi cant inhibition of Complex II and Complex IV activities in sepsis (**Fig. 4, *C*** and ***D***; and Supplemental Fig. 4, Supplemental Digital Content 6, http://links.lww.com/CCM/E503 legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). Vagus nerve stimulation completely prevented the suppression of Complex II and Complex IV activities (Fig. 4, *C* and *D*).

The WBC counts were reduced during sepsis, and vagus nerve stimulation did not affect this reduction (**Fig. 5*A***). The relative neutrophil and lymphocyte counts were not affected by sepsis nor vagus nerve stimulation (not shown). The relative CD14 monocyte counts were reduced in sepsis, and vagus nerve stimulation did not counteract this effect (**Supplemental Fig. 5**, Supplemental Digital Content 7, http://links.lww.com/CCM/E504; legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). The relative counts of activated CD14/swine leukocyte antigen-DR (SLA-DR) monocytes were reduced in sepsis and normalized upon vagus nerve stimulation (**Fig. 5*B***). The plasma levels of proin ammatory cytokines

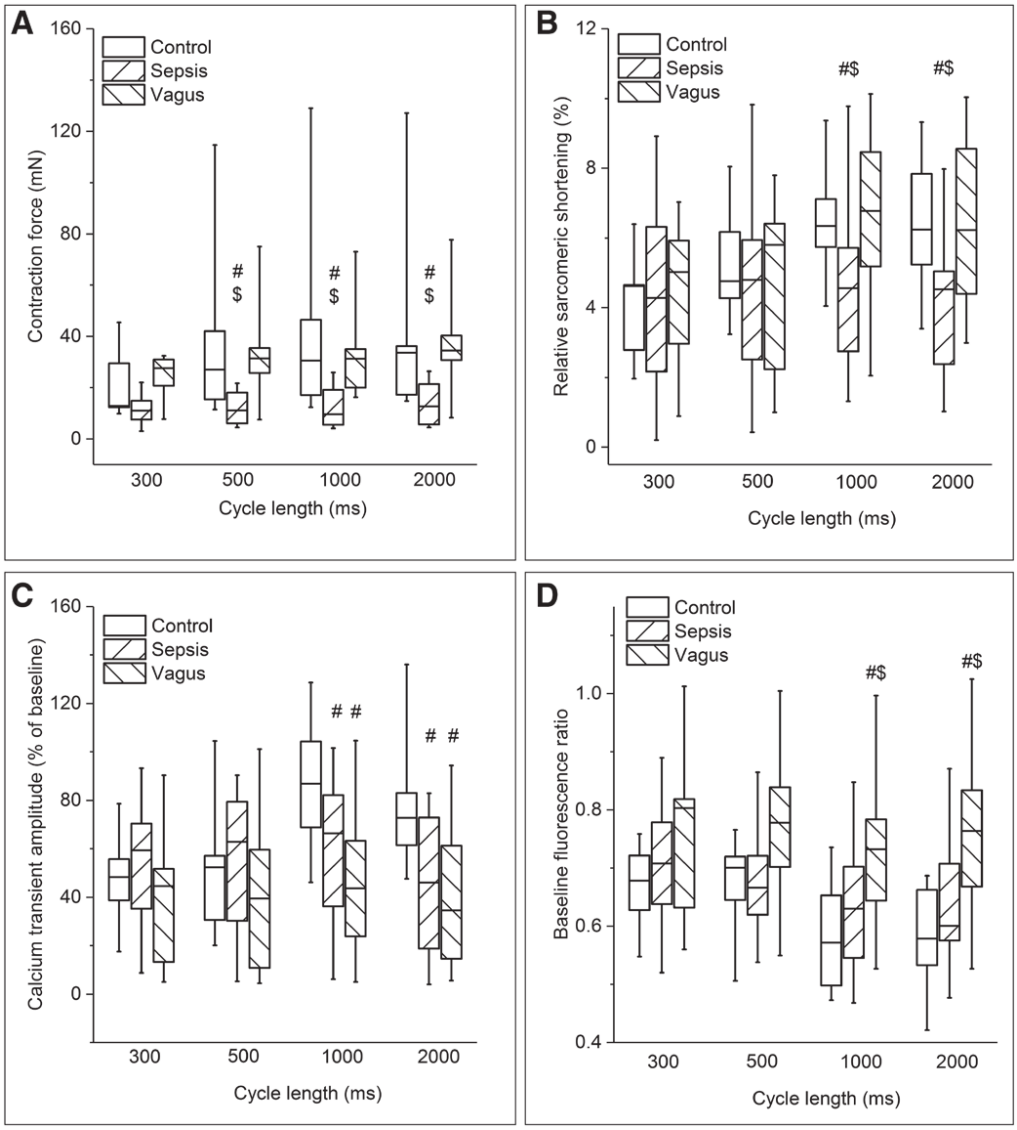

Figure 3. Contractility in cardiac trabeculae and isolated myocytes. **A**, Contraction force in cardiac trabeculae from control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at cycle lengths of 300, 500, 1,000 and 2,000 ms. **B**, Relative sarcomeric shortening (expressed in % of baseline) in isolated cardiac myocytes from control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at cycle lengths of 300, 500, 1,000 and 2,000 ms. **C**, Calcium transient amplitude (expressed in % of baseline) in isolated cardiac myocytes from control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at cycle lengths of 300, 500, 1,000 and 2,000 ms. **D**, Baseline uorescence ratio in isolated cardiac myocytes from control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at cycle lengths of 300, 500, 1,000 and 2,000 ms. #p < 0.05 versus control; \$$p$ < 0.05 sepsis versus sepsis + vagal stimulation. Data are presented as median with interquartile range (*box*) and total range (*whiskers*).

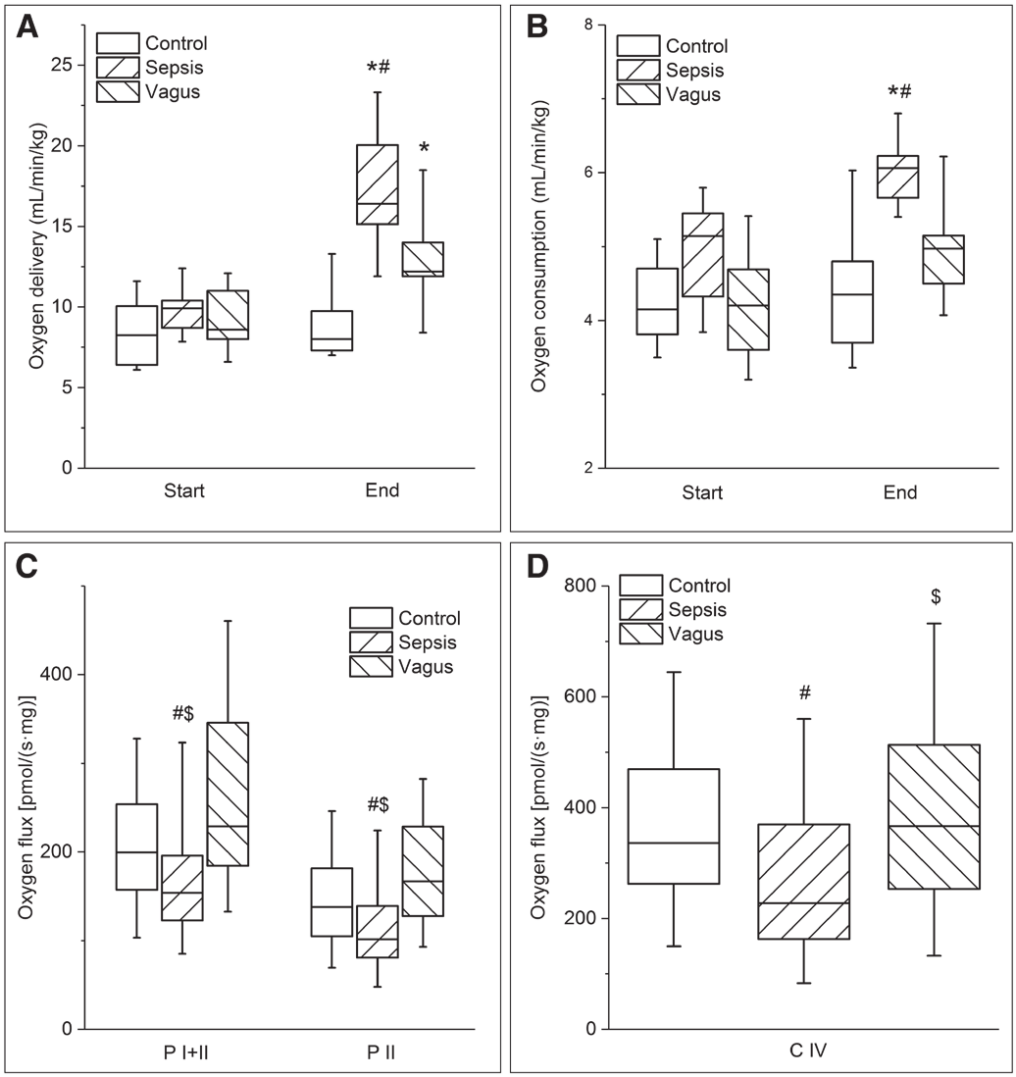

Figure 4. Oxygen delivery and consumption and mitochondrial function. **A**, Oxygen delivery in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **B**, Oxygen consumption in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **C**, Oxygen consumption in the presence of substrates for Complex I and II (P I + II), in the presence of substrates for Complex I and II and inhibitor of Complex I (P II) in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*). **D**, Complex IV (C IV) oxygen consumption in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*). **p* < 0.05 versus start; #*p* < 0.05 versus control; $*p* < 0.05 sepsis versus sepsis + vagus stimulation. Data are presented as median with interquartile range (*box*) and total range (*whiskers*).

tumor necrosis factor- , interleukin (IL)±6, and IL-8 signi cantly increased in sepsis regardless of vagus nerve stimulation (**Fig. 5C**; and Supplemental Fig. 5, Supplemental Digital Content 7, http://links.lww.com/CCM/E504 legend, Supplemental Digital Content 8, http://links.lww.com/CCM/E505). The plasma levels of anti-in ammatory cytokine IL-10 were below the detection limit (3 pg/mL) in all pigs.

DISCUSSION

In this study, the concept of the cholinergic anti-in ammatory pathway was tested. To the best of our knowledge, this was the rst time this pathway was tested in a large animal model of sepsis, and the positive results indicate the signi cant translational potential of vagus nerve stimulation and promising clinical implications. In resuscitated porcine progressive sepsis, the vagus nerve stimulation was associated with a number of bene cial effects, especially with respect to the cardiovascular system and energy metabolism, and consequently, vagus nerve stimulation signi cantly attenuated multiple organ dysfunction and reduced vasopressor requirements. The vagus nerve stimulation partially or completely prevented the development of hyperdynamic circulation, cellular myocardial depression, hyperlactatemia, the shift in the sympathovagal balance toward sympathetic dominance, and cardiac mitochondrial dysfunction and reduced the number of activated monocytes. The data suggest a signi cant therapeutic potential of vagus nerve stimulation for conditions related to sepsis and septic shock in a large animal model.

Septic hyperdynamic circulation was inhibited by the vagus nerve stimulation; only a slight increase in cardiac output and maintained stroke volume were observed in stimulated animals. On the contrary, in septic animals, high drive tachycardia that led to decreased stroke volume was observed. The hypothesis that decreasing the sympathetic effect on the heart may be bene cial in patients with septic shock has been already stated and tested by the use of beta-blockers (18) or ivabradine (19). A direct central parasympathetic effect induced by vagal stimulation might provide another way to decrease demands on the cardiovascular system and protect the heart. The dominant mode of action of vagus nerve stimulation, however, remains unclear. Low frequency (1±10 Hz) stimulation should preferentially activate vagal efferents (20), which suggests a direct cardiac effect of vagus nerve stimulation in our experimental conditions. Analysis of heart rate variability also showed a parasympathetic shift of the sympathovagal balance after vagus

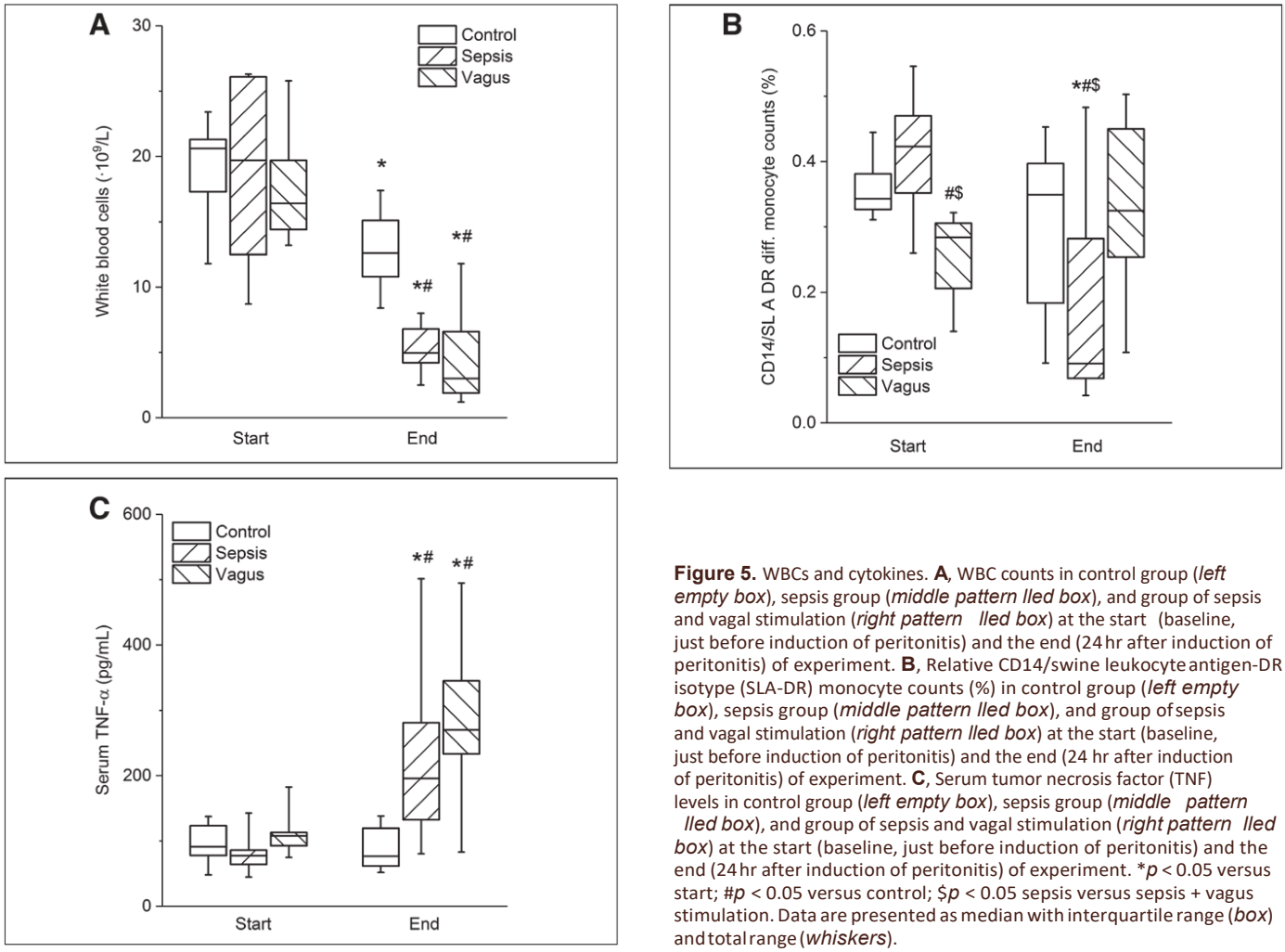

Figure 5. WBCs and cytokines. **A**, WBC counts in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **B**, Relative CD14/swine leukocyte antigen-DR isotype (SLA-DR) monocyte counts (%) in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. **C**, Serum tumor necrosis factor (TNF) levels in control group (*left empty box*), sepsis group (*middle pattern lled box*), and group of sepsis and vagal stimulation (*right pattern lled box*) at the start (baseline, just before induction of peritonitis) and the end (24 hr after induction of peritonitis) of experiment. *$p < 0.05$ versus start; #$p < 0.05$ versus control; \$$p < 0.05$ sepsis versus sepsis + vagus stimulation. Data are presented as median with interquartile range (*box*) and total range (*whiskers*).

nerve stimulation; however, a central autonomic effect due to afferent stimulation cannot be excluded. Since the vagus nerve contains both afferent and efferent bers (afferent bers representing over 80% of the nerve [21]), it is virtually impossible to distinguish afferent and efferent mechanisms of vagus nerve stimulation, which probably in uence each other in a complex re exive manner (22). Besides, the other observed effects of vagal stimulation (decrease in oxygen consumption, retained systemic vascular resistance, etc.) are dif cult to adjudge to cardiac effects only.

Interestingly, pigs with comparable cardiac preload that were treated with vagal nerve stimulation required lower amounts of resuscitation uid and less norepinephrine doses to achieve the hemodynamic targets. Taken together, we speculate that vagal nerve stimulation improves vasomotoricity and ameliorates the sepsis-induced vascular barrier dysfunction that is associated with microvascular uid leakage. Although the exact mechanism cannot be inferred from our study, our results suggest that the basis for improved vascular functions may be attributable to the restoration of cellular energy metabolism.

In this study, cardiac mitochondrial respiration was impaired in sepsis and normalized by vagus nerve stimulation. Although impaired mitochondrial oxygen consumption is frequently mentioned as a crucial factor that contributes to the pathogenesis of (multiple)organ dysfunction (23), studies performed in various rodent models of sepsis showed unchanged, decreased, or even increased mitochondrial respiration in the heart (24). Rare data on porcine fecal peritonitis models did not show any signi cant differences in mitochondrial oxygen consumption between control and vigorously resuscitated (including antibiotics) piglet groups (25). It is well known that factors such a particular sepsis models, animal species, disease severity and phase, and the experimental set-up can substantially impact variability of experimental results (26). Our study shows that sepsis-induced myocardial depression was associated with mitochondrial respiratory dysfunction, which corresponds well with the decreased oxygen extraction ratio and together suggests that in this porcine model, the mitochondrial dysfunction is not limited to cardiac tissue. To prove this hypothesis, however, mitochondrial studies in other than cardiac tissues will be necessary. Furthermore, the fact that in sepsis the oxygen delivery increased more than oxygen consumption suggests that mitochondrial dysfunction might be the primary organ dysfunction event. In this study, the suppression

of mitochondrial respiration was dominantly due to the inhibition of Complex II and Complex IV. In contrast to studies in rodents (27), the activity of Complex I was not affected.

The impact of vagus nerve stimulation on mitochondrial oxygen consumption by cardiac tissue has not been studied. However, some data indicate that cardioprotective effects of vagus nerve stimulation might be at least partly mediated by improved mitochondrial functions (28). For example, the bene cial effects of vagus nerve stimulation on mitochondria have been documented in a porcine model of myocardial ischemia, where the vagus nerve stimulation attenuated cardiac mitochondrial reactive oxygen species production, depolarization, and swelling (29). A recently-published study suggested that 7-nicotinic acetylcholine receptor could be involved in the protection against lipopolysaccharide-induced sepsis myocardial injury and apoptosis in mice (30). The present data indicate that vagus nerve stimulation improves mitochondrial respiration and cardiac metabolism.

The peritonitis-induced sepsis porcine model was associated with myocardial depression, as evidenced by decreased stroke volume, reduced contraction force of cardiac trabeculae, and reduced sarcomeric shortening in isolated cardiac myocytes. The vagus nerve stimulation completely reversed all of these manifestations of septic myocardial depression. In septic cardiac myocytes, the reduction of sarcomeric shortening was associated with decreased calcium transient. The vagus nerve stimulation did not affect the amplitude of calcium transient but led to elevated baseline intracellular calcium levels, which could contribute to the normalization of cardiac contractile forces. Furthermore, higher intracellular calcium levels might contribute to the improved mitochondrial function that was observed upon vagus nerve stimulation. The cardiac sarcoplasmic reticulum and mitochondria form a mitochondrial calcium microdomain (31), and mitochondrial calcium uptake stimulates the tricarboxylic acid cycle and adenosine triphosphate production (32).

Among the multiple bene cial effects of vagus nerve stimulation, it is dif cult to determine the primary effect. The lack of effect on the plasma levels of cytokines argues against the dominant role of upstream anti-in ammatory mechanisms, although the reversal of the reduced CD14/SLA-DR monocyte count might be of importance. The reduction of CD14/SLA-DR monocytes in sepsis probably re ects the transition of steady-state monocytes to in ammatory monocytes that lack SLA-DR (33). Although the vagus nerve stimulation suppressed this effect of sepsis on monocyte phenotype, it did not prevent an increased production of in ammatory mediators. The absence of an effect of vagus nerve stimulation on cytokine production in porcine progressive sepsis stands in stark contrast to the cytokine inhibition that was observed in rodent sepsis models (10) and suggests that signi cant species differences in cholinergic anti-in ammatory mechanisms exist.

The translation of vagus nerve stimulation to the clinical level should be feasible since vagus nerve stimulation has already been approved by regulatory bodies of the United States, European Union, and Canada as a treatment for epilepsy and depression. Furthermore, vagus nerve stimulation is considered a safe technique, and few side effects are observed at the low frequencies that are classically used in in ammatory conditions (20). An implantable device for direct vagus nerve stimulation that includes spiral electrodes and a battery-powered pulse generator is commercially available. As an alternative noninvasive option, transcutaneous vagus nerve stimulation has been demonstrated to be a safe and well-tolerated method in patients with pharmacoresistant epilepsy (34).

STUDY LIMITATIONS

Vagus nerve stimulation was initiated 6 hours after the induction of peritonitis when the sepsis was not fully developed. The ef cacy of the vagus nerve stimulation when applied in later stages of sepsis progression or during septic shock remains to be examined.

Our experimental design and subsequent in vitro analysis of tissues and cells precluded the analysis of mortality and/or recovery from the disease. Future studies will be needed to determine the potential mortality bene t for this therapeutic option.

Different norepinephrine requirements might contribute to the results reported and complicate their interpretation. Although norepinephrine was administered secondarily in response to a drop in blood pressure, possible contributions of norepinephrine to sympathovagal balance through various complex feedback loops cannot be excluded and will require further clari cation.

The effects of sepsis and of vagus nerve stimulation were only studied in the rst 24 hours of sepsis, and therefore, the potential long-term implications of the disease and of the intervention remain unclear. To reach the stage of refractory septic shock within 24 hours from induction of peritonitis, antibiotic therapy must be omitted, which might represent a potential shortcoming of the model clinical relevance. Possible interactions of the antibiotic therapy and vagus nerve stimulation represent another important question, which will warrant further investigation.

CONCLUSIONS

In a clinically-relevant porcine model of sepsis and septic shock, vagus nerve stimulation was associated with a number of bene cial effects that resulted in signi cantly attenuated multiple organ dysfunction and reduced vasopressor and uid resuscitation requirements. Vagus nerve stimulation might provide a signi cant therapeutic potential in a large animal model of progressive septic shock.

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Evaluation of Mesenchymal Stem Cell Therapy for Sepsis: A Randomized Controlled Porcine Study

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Background: Treatment with mesenchymal stem cells (MSCs) has elicited considerable interest as an adjunctive therapy in sepsis. However, the encouraging effects of experiments with MSC in rodents have not been adequately studied in large-animal models with better relevance to human sepsis.

Objectives: Here, we aimed to assess safety and ef!cacy of bone marrow-derived MSCs in a clinically relevant porcine model of progressive peritonitis-induced sepsis.

Methods: Thirty-two anesthetized, mechanically ventilated, and instrumented pigs were randomly assigned into four groups (*n* 8 per group): (1) sham-operated group (CONTROL); (2) sham-operated group treated with MSCs (MSC-CONTROL); (3) sepsis group with standard supportive care (SEPSIS); and (4) sepsis group treated with MSCs (MSC-SEPSIS). Peritoneal sepsis was induced by inoculating cultivated autologous feces. MSCs (1 10^6/kg) were administered intravenously at 6 h after sepsis induction.

Results: Before, 12, 18, and 24 h after the induction of peritonitis, we measured systemic, regional, and microvascular hemodynamics, multiple-organ functions, mitochondrial energy metabolism, systemic immune-in[gammatory response, and oxidative stress. Administration of MSCs in the MSC-CONTROL group did not elicit any measurable acute effects. Treatment of septic animals with MSCs failed to mitigate sepsis-induced hemodynamic alterations or the gradual rise in Sepsis-related organ failure assessment scores. MSCs did not confer any protection against sepsis-mediated cellular myocardial depression and mitochondrial dysfunction. MSCs also failed to modulate the deregulated immune-in[gammatory response.

Conclusion: Intravenous administration of bone marrow-derived MSCs to healthy animals was well-tolerated. However, in this large-animal, clinically relevant peritonitis-induced sepsis model, MSCs were not capable of reversing any of the sepsis-induced disturbances in multiple biological, organ, and cellular systems.

Keywords: sepsis, septic shock, acute organ dysfunction, mesenchymal stem cells, cell therapy, immunomodulation

INTRODUCTION

The lack of e[]ective therapy for sepsis remains a major unmet medical need. Even though substantial progress has been made in understanding the underlying pathophysiology of sepsis, translation of these advances into clinically e[]ective therapies has been disappointing. Given the extreme complexity of sepsis pathogenesis, the paradigm "one disease, one drug" is obviously awed and combinations of multiple targets that inde early immunomodulation and cellular protection are needed. The immunomodulatory, anti-in ammatory, anti-apoptotic, metabolomic, and anti-microbial e[]ects of mesenchymal stem cells (MSCs) may have scienti c and clinical relevance in this context (1). Indeed, application of MSCs in preclinical models of sepsis has been associated with lower mortality, improved course of sepsis due to inhibition of pro-active elements of the immune system, and a change in the pro- and anti-cytokine ratio both in *vitro* and *in vivo* (1–3). In addition, no preclinical study published so far has demonstrated adverse e[]ects associated with the application of MSCs in animal models of sepsis. It must be emphasized, however, that these encouraging results were largely derived from rodent models with clearly limited relevance to human sepsis. Hence, a thorough investigation of the e[]ects of MSCs in clinically relevant large-animal models is urgently needed before translation to the clinical eld. Therefore, we conducted a randomized controlled experimental study to explore the biological e[]ects of MSCs on the background of standard care in comparison to standard conservative therapy in a porcine model of peritonitis-induced progressive sepsis. The model ful lls recently de ned requirements for preclinical s studies (4). We aimed to examine both the safety of MSCs in healthy animals and the e[]ect of MSCs on various biological systems related to multiple pathophysiological pathways during sepsis progression.

MATERIALS AND METHODS

Mesenchymal Stem Cells

Allogenic porcine MSCs were isolated from healthy pigs. Bone marrow from the tibia or femur bones was aspirated into 50-mL tubes (Techno Plastic Products-TPP, Trasadingen, Switzerland) containing heparin (B Braun) by puncture with a sterile needle. MSCs were isolated from bone marrow by gradient centrifugation (440 *g*, 30 min) on Ficoll-Paque Plus (GE Healthcare, North Richland Hills, Texas, USA). The layer of mononucleated cells was washed with phosphate-bu[]ered saline (PBS) and plated in a 75-cm^2 culture ask (TPP) containing -MEM cell culture medium (Thermo Fisher Scienti c, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scienti c), 1 mM L-glutamine (Biochrom, Cambridge, UK), 6.0 mg/mL penicillin/10 mg/mL streptomycin (Biosera, Nuaille, France), and 0.25 mg/mL gentamicin (Biosera). Culture medium was changed every second day. After 10 days, MSCs were harvested by ethylenediaminetetraacetic acid (EDTA)/trypsin 1 (Biosera) and separated into three 75-cm^2 culture asks (TPP). Culture medium was changed again every second day, and after 10 days, MSCs were harvested by EDTA/trypsin 1 (Biosera) and cryopreserved in liquid nitrogen (1 10^6 cells/cryotube). Four weeks before transplantation, MSCs were thawed, plated in 150-cm^2 asks (TPP) containing 20 mL of the culture medium described above, and cultured for 4 weeks to obtain about 5 10^7 cells with one passage cycle. In this way, the stem cell properties of MSCs were maintained. On the day of transplantation, MSCs were harvested as described above, counted, re-suspended in 100 mL of saline solution (B Braun) pre-warmed to 37 C (10^6/kg of pig weight) per pig, and immediately administered through the central venous line 6 h after induction of peritonitis. Before transplantation, the stem cell phenotype of MSCs was evaluated by ow cytometric detection of CD90, CD73, and CD44 markers (shown in **Supplemental Digital Material**). MSCs were washed with PBS and stained with 5 L of APC-CD90 (Biolegend, San Diego, CA, USA), PE-CD73 (Biolegend), BV421-CD44 (Biolegend), and FITC-CD45 (Bio-Rad, Hercules, CA, USA) for 15 min in the dark at room temperature. Afterwards, MSCs were washed and resuspened in 300 L of PBS followed by measurement on a BD FACS Aria Fusion cell analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). Post-acquisition analysis of data was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA). The ability of transplanted MSCs to di[]erentiate was evaluated by their formation of adipo-, osteo-, and chondro-lineages. MSCs were seeded into 12-well-cultivation dishes (TPP) with a seeding density of 3.8 10^4 cells/well for adipogenic and chondrogenic di[]erentiation, and 1.9 10^4 cells/well for osteogenic di[]erentiation in culture medium. After a 24-h attachment period, the medium was discarded and replaced with

Abbreviations: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CVP, Central venous pressure; DO_2 , Systemic oxygen delivery; EDTA, Ethylenediaminetetraacetic acid; hsTnT, High-sensitivity troponin T; IL-6, Interleukin 6; IL-8, Interleukin 8; IL-10, Interleukin 10; MAP, Mean arterial pressure; MSCs, Mesenchymal stem cells; PAOP, Pulmonary artery occlusion pressure; PaO_2/FiO_2, Oxygenation index; PBS, Phosphate-bu[]ered saline; SOFA, Sepsis-related organ failure assessment; TNF- , Tumor necrosis factor ; VO_2 , Systemic oxygen uptake.

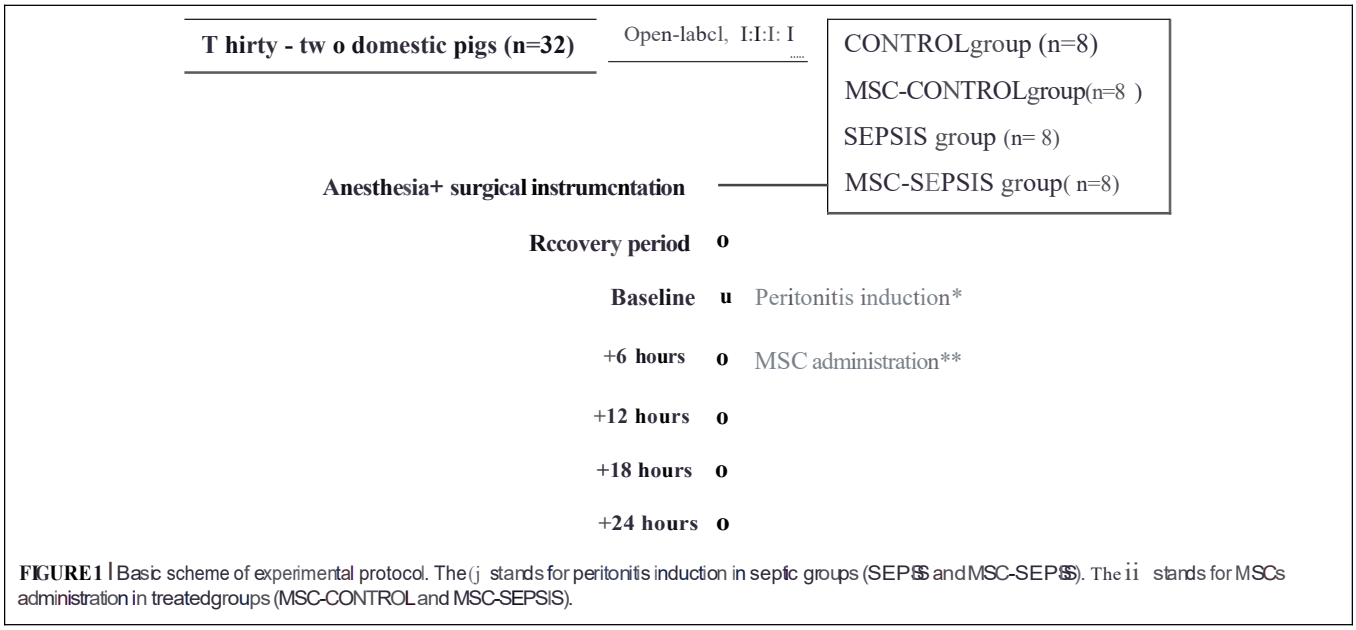

FIGURE 1 | Basic scheme of experimental protocol. The (j stands for peritonitis induction in septic groups (SEPSIS and MSC-SEPSIS). The ii stands for MSCs administration in treatedgroups (MSC-CONTROL and MSC-SEPSIS).

3mL of StemPro® Adipogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit, or StemP ro® Osteogenesis Differentiation Kit (all ThermoFisher Scientific) for adipogeni,c chondrogenic, or osteogenic differentiation, respectively. After a differentiation period of 21 days, the cells were stained with oil red O for lipid droplet visualization in adipogenesis, alcian blue for glycoprotein visualization in chondrogenesi,s and alizarin red S (all Sigma Aldrich, St. Louis, MO, USA) for calcium ion visualization in osteogenesi.s Donor MSCs were not matched with recipients.

Animals

Ali experiments were performed in adherence to the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU). The protocols were approved by the Committee for Experiments on Animals of the Faculty of Medicine, Charles University, in Pilsen and by the Ministry of Education, Youth and Sports of the Czech Republic (protocol no. MSMT-20064/2015-3). Ali experiments were performed in the Laboratory of Experimental Intensive Care Medicine of the Biomedical Center at the Faculty of Medicine in Pilsen. Thirty-two domestic pigs (breed Black Pied Prestice Pig) from conventional breeding facility (ZD Mladotice, Czech Republic) of either sex with a median weight of 43.5 (40- 46) kg were used.

Experimental Protocol

The animals were assigned to one of four experimental groups (at a ratio of 1:1:1:1): sham-operatedcontrol group (CONTROL, $n = 8$), control group treated with MSCs (MSC-CONTROL, $n = 8$), sham-operated sepsis group (SEPSIS, $n = 8$), and septic group treated with MSCs (MSC-SEPSIS, $n = 8$). Theintervention was open-labeled.In septic animals, peritonitis was induced by inoculating 1 g/kg of autologous feces (collected preoperatively and suspended in 200 mL of isotonic saline at 38°C) into the abdominal cavity followed by a 6-h recovery period (baseline). When sepsis-associated hypotension developed, fluid boluses (10 ml/kg of Ringerfundin solution) were administered in a goal-directed manner guided by filling pressures and cardiac output response as part of hemodynamic resuscitation. Fluid resuscitation was discontinued if there was no further increase in cardiac output (10% threshold) and/or when the pulmonary artery occlusion pressure (PAOP) reached more than 15 mmHg. Continuous infusion of norepinephrine was adrninistered if the mean arterial pressure (MAP) fell below 65 mmHg and no further positive hemodynamic response was elicited via fluid resuscitation. Norepinephrine was titrated to maintain MAP between 65 and 70 mmHg. In MSC-CONTROL and MSC-SEPSIS groups, MCSs were infused in a clinically relevant <lose (1×10^6/kg) over 10 min via the centra! venous line 6 h from the baseline. The MSC <lose was chosen on the basisof several previous clinical (5, 6) as well as experimental rodent (1, 2) and largeanimal studies(7). At theendof theexperiment, theanimals were euthanized by anesthetic overdose and excision of the heart. Experimental protocol scheme isshown in **Figure 1.**

Anesthesia and Surgical Instrumentation

Ali animals were anesthetized with xylazine (1 mg/kg) and tiletamin-zolazepam (5 mg/kg). A 2 mg/kg <lose of 2% propofol was administered after intravenous line insertion. Animals were intubated and mechanically ventilated as follows: volumecontrol mode with tidal volume of 8- 10 mL/kg, positive end-expiratory pressure of 0.6 kPa, Fi02 of 0.3, and respiratory rate adjusted to maintain arterial normocapnia. During surgery, anesthesia was maintained by continuous administration of 2% propofol (4- 6 mg/kg/h), fentanyl (8- 10 µ,g/kg/h), and rocuronium (2 mg/kg/h). Drug dosing was halved after surgery. Continuous infusion of Ringerfundin solution (B-Braun Melsungen AG,

Melsungen, Germany) was used as a fluid replacement at a dose of 10 mL/kg/h during surgery and 7 mL/kg/h thereafter. Continuous infusion of 10% glucose served to maintain arterial blood normoglycemia.

Before the surgical procedure, an arterial catheter was placed in the femoral artery for continuous invasive blood pressure monitoring and blood sampling. Pulmonary artery and central venous catheters were introduced via external jugular veins under ultrasound guidance. Midline laparotomy was performed and a pre-calibrated ultrasound flow probe (Transonic Systems, Ithaca, NY, USA) was placed around the left renal artery. Double-lumen ileostomy was constructed to assess gut mucosal microcirculation. Peritoneal drainage was inserted and epicystostomy was performed prior to abdominal wall closure. A recovery period of 6 h followed the surgical procedures.

Monitoring, Sampling, and Measurements

Data sets were recorded at baseline/sepsis induction, 12, 18, and 24 h after peritonitis induction. Measurements and calculations included the assessment of systemic and regional hemodynamics (see **Supplemental Digital Material**),

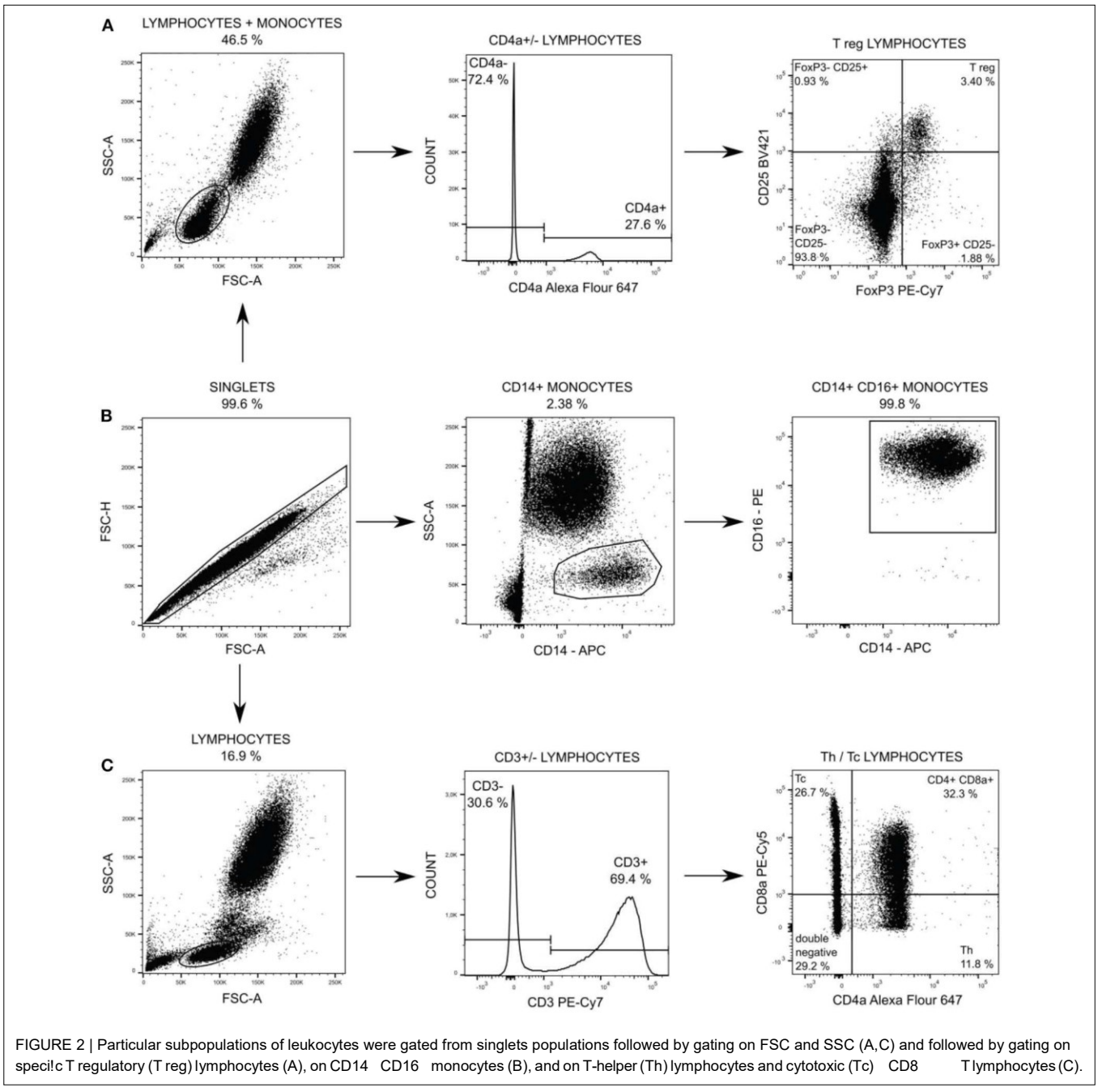

FIGURE 2 | Particular subpopulations of leukocytes were gated from singlets populations followed by gating on FSC and SSC (A,C) and followed by gating on specific T regulatory (T reg) lymphocytes (A), on CD14 CD16 monocytes (B), and on T-helper (Th) lymphocytes and cytotoxic (Tc) CD8 T lymphocytes (C).

uids, and vasopressor requirement. Arterial blood samples were analyzed for lactate, arterial and mixed venous blood gases, pH, base excess by using POCT analyzer (Cobas B 123, Roche, Diagnostics, USA). Complete blood count and other biochemical analyses included serum creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-sensitivity troponin I (hsTnI), albumin, and total protein were performed as described previously (8). Cytokines, including IL-6, IL-8, IL-10, and tumor necrosis factor (TNF-), as well as C-reactive protein were analyzed by using the ELISA method (Porcine Quantikine ELISA Kit, R&D System, Minneapolis, USA). Oxidative stress biomarker 8-isoprostane analysis was performed by using EIA porcine kit (Cayman Chemical, Michigan, USA). In addition, basic hemodynamics and acid-base balance parameters were measured prior to MSC's administration (i.e., 6 h, data not shown) to capture sepsis development.

The modi ed sepsis-related organ failure assessment (SOFA) score was determined according to the Third international consensus de nitions for sepsis and septic shock (9) with exclusion of the Glasgow coma scale-based neurologic component. Gut mucosal microcirculation was recorded at each time point using a MicroScan handheld video microscope (MicroVision Medical, Amsterdam, Netherlands). Each record was split into three parts to visualize di]erent areas of the gut mucosa and analyzed by Automated Vascular Analysis software version 4.0 (MicroVision Medical).

Cardiac myocytes were isolated from the left ventricle by enzymatic dissociation with collagenase A (Sigma-Aldrich) as previously reported (8). Sarcomeric contractions of isolated cardiac myocytes were measured with the HyperSwitch Myocyte Calcium and Contractility System (IonOptix LLC, Westwood, MA, USA), with the Sarclen sarcomere length acquisition module. Measurements were performed in normal Tyrode solution at 37 0.5 C. Cells were stimulated with the MyoPacer Field Stimulator (IonOptix LLC) at cycles of 300, 500, 1,000, and 2,000 ms. IonWizard 6.5 software (IonOptix LLC) was used for o[i<ine analysis.

Cardiac mitochondrial function was assessed using high-resolution respirometry (oxygraph Oroboros O2k; Oroboros Instruments, Innsbruck, Austria). Mitochondrial oxygen consumption was measured in permeabilized left ventricular samples at 37 C. Transmural sample (1 cm^3) was dissected from the proximal free wall of the left ventricle and cut into 2 mg tissue samples that were quickly transferred into ice-cold biopsy preserving solution (BIOPS: 10 mM Ca-EGTA bu[)er, 0.1 M free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) with saponin (50 g/ml), shaken gently on ice for 30 min, washed in mitochondrial respiration medium (MiR06: 0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM D-sucrose, 1 g/l albumin essentially fatty acid free, and 280 u/ml catalase) for 10 min and then placed into

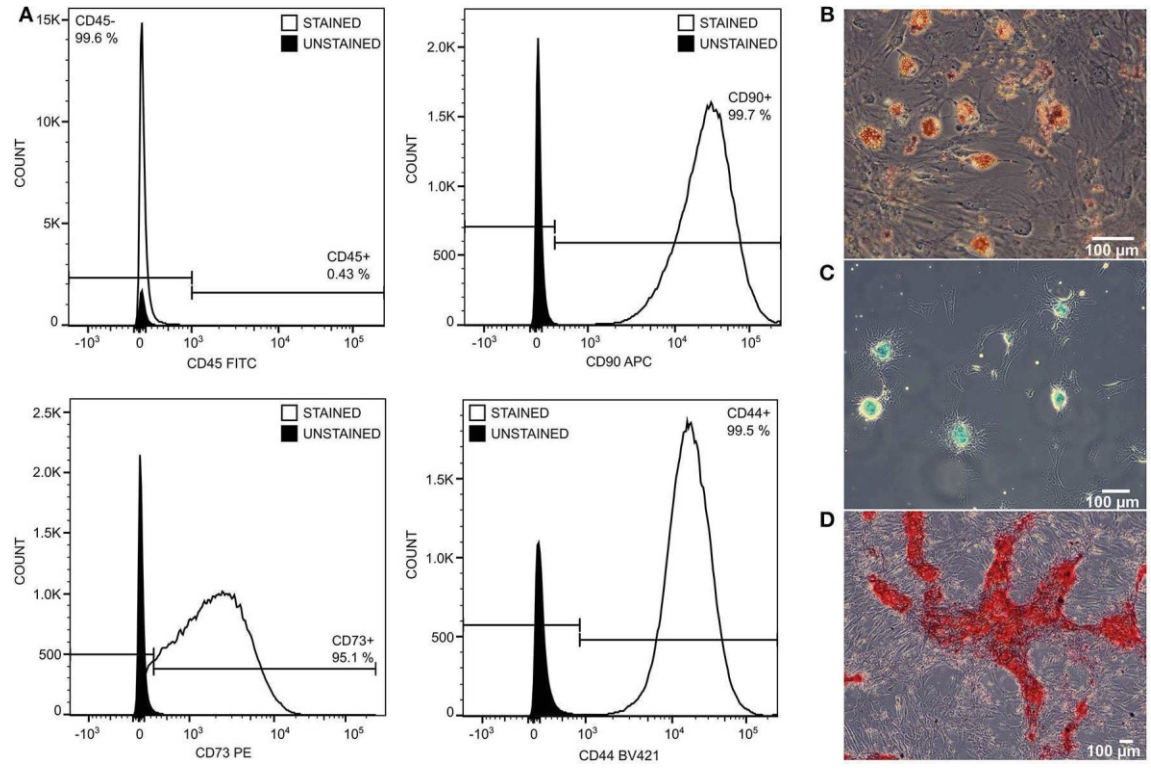

FIGURE 3 | The quality of transplanted MSCs was monitored by expression of stem cell surface markers and by differentiation ability. MSCs were negative to CD45 and positive to CD90, CD73, and CD44 (A). They differentiate into adipo- (B), chondro- (C), and osteo- (D) lineage in 21 days of differentiation protocol.

oxygraph chambers. In the titration protocol, several substrates and inhibitors of the mitochondrial respiratory system were sequentially added into the chambers to determine particular respiratory states and activities of mitochondrial respiratory complexes. Oxygen consumption was analyzed online by DatLab software (Oroboros Instruments) as the negative time derivative of oxygen concentration in the chamber.

Flow Cytometry of Leukocytes Subpopulations

Changes in leukocytes subpopulations were monitored by ow cytometry. One hundred microliter of EDTA treated blood was stained for 15 min in dark and room temperature by cocktail of anti-CD speci c antibodies (Table antibodies, **Supplemental Digital Material**) at baseline, 12 and 18 h after peritonitis induction (24 h was not measured due to operational reasons). Afterward staining, all samples were lysed by BD FACS Lysing Solution (Becton Dickinson, San Jose, USA) to separate leukocytes and contaminating erythrocytes. CD14posCD16pos monocytes, T-helper (Th) and cytotoxic T (Tc) CD8 T lymphocytes were washed by PBS, pelleted (300 g, 5 min) and re-suspened in 300 l of PBS followed by measurement. T regulatory (Treg) lymphocytes were xed and permeabilized by FoxP3 staining bu]er set (Thermo Fisher Scienti c), washed by PBS, pelleted (300 g, 5 min) and re-suspened in 300 l of PBS followed by measurement. The measurement was performed by the BD FACS Aria Fusion cell analyzer (Becton Dickinson). One million events was acquired and the post-acquisition analysis of data was performed using FlowJo software (Becton Dickinson). The gating strategy for each subpopulation is summarized in **Figure 2**. Absolute counts of particular subpopulations were determined from total leukocytes counts acquired within standard biochemical analysis of blood samples.

Statistical Analysis

Statistical analysis was carried out using SigmaStat software version 3.5 (Systat Software Inc., Erkrath, Germany). Results are presented as median (interquartile range, range). Statistical comparisons were made using non-parametric statistics. Di]erences within each group before and after induction of sepsis were tested using Friedman ANOVA on ranks and, subsequently, Dunn's test for multiple comparisons. The Mann-Whitney rank sum test was performed to compare data between treatment groups (CONTROL vs. MSC-CONTROL; SEPSIS vs. MSC-SEPSIS). A $p < 0.05$ was regarded as statistically signi cant.

RESULTS

Administrated MSCs achieved standard quality as determined by the expression of stem cell markers CD90 , CD73 , CD44 , CD45 , and by their ability to di]erentiate into adipo-, chondro-, and osteo-lineages (**Figure 3**).

Five experiments were repeated due to perioperative death (n 2) or premature death before baseline data collection or treatment commencement (n 3). Data from animals that

TABLE 1 | Parameters describing organ function and systemic in[gammation in sham-operated animals with and without MSC administration.

Parameter	Timepoint	CONTROL	MSC-CONTROL
Urea (mmol/L)	Baseline	5 (4.2–5.5)	5.7 (5–6.6)
	12 H	5.5 (3.6–5.9)	6.1 (5.1–7.3)*
	18 H	5 (3.3–5.6)	5.5 (4.5–6.6)
	24 H	4.4 (3.2–4.6)	4.7 (3.8–5.8)*
Creatinine (mol/L)	Baseline	95 (80–103)	99 (87–110)
	12 H	93 (77–108)	100 (88–107)
	18 H	88 (77–102)	95 (86–102)
	24 H	85 (68–93)	89 (83–103)
AST (kat/L)	Baseline	0.7 (0.6–1.5)	0.8 (0.6–1.9)
	12 H	0.8 (0.7–1.4)	0.9 (0.8–1.1)
	18 H	0.8 (0.7–1.6)	1.0 (0.9–1.5)
	24 H	0.8 (0.7–1.4)	1.0 (0.9–1.4)
ALT (kat/L)	Baseline	0.5 (0.5–0.7)	0.6 (0.5–0.7)
	12 H	0.5 (0.5–0.6)	0.5 (0.48–0.51)*
	18 H	0.5 (0.4–0.6)	0.5 (0.47–0.50)*
	24 H	0.5 (0.4–0.5)*	0.52 (0.5–0.5)*
Trombocytes (1 10^9/L)	Baseline	285 (226–315)	260 (231–357)
	12 H	227 (204–302)	200 (191–236)*
	18 H	236 (213–271)	201 (164–220)*
	24 H	241 (216–252)	194 (190–226)*
PaO_2/FiO_2 (mmHg)	Baseline	459 (410–510)	450 (441–466)
	12 H	408 (399–460)	452 (410–455)
	18 H	344 (262–404)*	355 (327–414)*
	24 H	272 (232–353)*	313 (187–412)*
hsTnT (ng/L)	Baseline	7.2 (6.2–18.1)	9.7 (7.4–36.3)
	12 H	7.7 (6.7–21.6)	9.2 (7.6–24.8)
	18 H	6.1 (5.6–17.4)*	9 (7.1–19.9)
	24 H	5.9 (4.5–17.8)*	10.5 (6.6–18.7)
IL-6 (ng/L)	Baseline	47 (36–75)	59 (47–114)
	12 H	48 (30–63)	38 (16–64)
	18 H	64 (48–212)	61 (33–66)
	24 H	352 (139–1,451)*	175 (35–768)
TNF- (ng/L)	Baseline	91.3 (77.7–123.2)	100.3 (62.1–145.1)
	12 H	58.9 (54.2–89.5)	52.7 (38.8–75.8)*
	18 H	60.0 (53.4–68.5)	59.3 (50.7–88.9)*
	24 H	76.5 (61.5–119.3)	94.2 (57.4–112.2)
8-Isoprostane (g/L)	Baseline	32.0 (4.9–55.8)	43.3 (23.0–93.2)
	12 H	8.4 (6.7–28.5)	23.5 (11.6–31.3)
	18 H	5.7 (4.9–14.1)	15.1 (4.0–37.1)
	24 H	6.7 (4–72.3)	55.6 (14.1–89.4)

*AST, aspartate aminotransferase; ALT, alanine aminotransferase; PaO_2/FiO_2, oxygenation index; hsTnT, high-sensitivity troponin T; IL-6, interleukin 6; TNF- , tumor necrosis factor . *$p < 0.05$ between baseline and time-point. No statistical signi!cance was found between groups.*

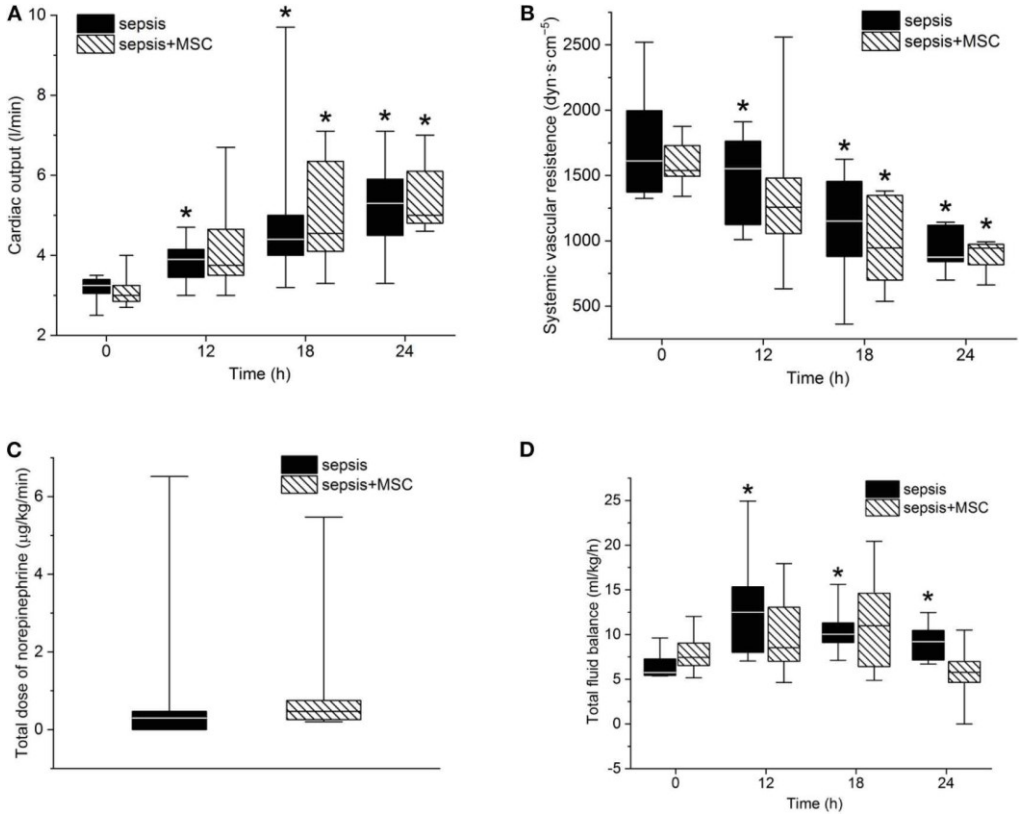

FIGURE 4 | (A) Cardiac output, systemic vascular resistance (B), total dose of norepinephrine (C) and total [guid balance (D). The (*) stands for signi!cant difference in time ($p < 0.05$ vs. baseline).

died prematurely were not used for analysis. Administration of MSCs to sham-operated healthy animals did not induce any signi[cant alterations in systemic, regional, or microvascular hemodynamics (data not shown). Similarly, neither target organ functions nor markers re[ecting in[ammatory status and oxidative stress were a[ected by MSCs **(Table 1)**.

All animals in both septic groups developed sepsis according to SEPSIS-3 criteria. Seven animals in the sepsis group and six animals in the MSC-SEPSIS group completed the whole 24-h protocol. Three animals (*n* 1 in sepsis, *n* 2 in MSC-SEPSIS group) died prematurely due to refractory septic shock. At the start of treatment (i.e., 6 h after induction of peritonitis) there were no statistically signi[cant di[erences in any measured variables between SEPSIS and MSC-SEPSIS groups (data not shown).

After the induction of peritonitis, all pigs developed hyperdynamic sepsis with an increased cardiac output and reduced systemic vascular resistance, without intergroup di[erences **(Figures 4A,B)**. Six pigs (75%) in the sepsis group and eight pigs (100%) in the MSC-SEPSIS group required vasopressor support to maintain MAP above 65 mmHg. The total dose of norepinephrine was comparable in both septic groups **(Figure 4C)** as was the time to the [rst administration of norepinephrine [1,093 (885–1,165) min in SEPSIS vs. 748 (594–944) min in MSC-SEPSIS group; *p* 0.345]. Likewise, there were no signi[cant di[erences in the amount of [uid administered for hemodynamic resuscitation **(Figure 4D)**. Other hemodynamic and metabolic variables are summarized in **Table 2**.

The modi[ed SOFA score progressively increased in both septic groups. Treatment with MSCs failed to attenuate sepsis-induced organ dysfunction. The tendency of the SOFA score to increase was even more pronounced in the MSC-SEPSIS group, mainly as a result of earlier initiation of norepinephrine administration **(Figure 5A)**. Indeed, no sign of a bene[cial e[ect of MSCs was observed even when single organ systems included in SOFA score (i.e., lungs, kidneys, liver, platelets) were evaluated separately (single organ data not presented).

Peritonitis-induced sepsis resulted in gradually increased plasma levels of TNF- and IL-6, providing evidence of a progressive systemic in[ammatory response **(Figures 5B,C)**. Treatment with MSCs did not result in a favorable e[ect on any of these variables. Plasma levels of IL-10 remained under the detection limit in all animals. Levels of CD14 CD16 monocytes, and Th, Tc CD8 T lymphocytes, and T_{reg} lymphocytes decreased in time due to sepsis in both experimental groups. The CD3 CD4a–CD8a CD8b gating consistently provided lower cell numbers than the CD3 CD4a–CD8a gating [50–70%, e.g., 69% (34%, 63%) in sepsis baseline, 62% (36%, 66%) in 18 h sepsis vs. 48% (36%, 64%) in sepsis MSC baseline, 60% (42%, 63%) in 18 h

TABLE 2 | Hemodynamic and metabolic variables in septic animals with and without MSC administration.

Parameter	TP	SEPSIS	MSC-SEPSIS
MAP (mmHg)	Baseline	73 (68–81)	70 (64–74)
	12 H	80 (70–87)	69 (66–75)
	18 H	71 (64–71)	68 (64–73)
	24 H	72 (69–73)	72 (68–75)
PAOP (mmHg)	Baseline	9 (8–9)	9 (6–10)
	12 H	11 (8–11)	8 (6–10)
	18 H	11 (10–13)	8 (7–10)
	24 H	10 (9–12)	9 (8–11)
CVP (mmHg)	Baseline	8 (8–10)	7 (7–9)
	12 H	11 (10–12)*	9 (8–12)
	18 H	12 (10–14)*	10 (8–14)*
	24 H	11 (10–15)*	12 (10–14)*
DO_2 (mL/min/kg)	Baseline	10.2 (9.1–11.0)	10.2 (9.0–10.7)
	12 H	12.2 (11.2–15.3)	14.4 (12.7–16.2)
	18 H	15.1 (12.4–17.9)	16.6 (14.8–19.5)
	24 H	16.7 (14.9–21.0)*	20.5 (18.7–26.5)*
VO_2 (mL/min/kg)	Baseline	5.3 (4.5–5.6)	5.1 (4.7–5.6)
	12 H	5.7 (4.9–6.4)	6.0 (5.4–6.8)
	18 H	5.7 (5.3–6.3)	6.7 (5.6–7.7)
	24 H	6.1 (5.7–6.4)	6.8 (6.6–8.1)*
RBF/CO (%)	Baseline	6.7 (5.5–10.5)	6.2 (5.4–7.5)
	12 H	5.3 (3.5–6.3)*	4.0 (2.2–5.0)
	18 H	4.6 (2.9–5.6)*	2.2 (0.9–4.1)*
	24 H	3.7 (2.1–5.4)*	3.0 (2.2–3.7)*
Total vessel density (gut mucosa) (mm/mm^2)	Baseline	16.0 (14.5–22.9)#	26.6 (22.6–28.7)#
	12 H	22.2 (13.1–24.2)	20.2 (16.0–32.8)
	18 H	21.4 (16.6–26.0)	19.4 (17.6–22.2)
	24 H	17.4 (12.0–27.6)	17.7 (15.1–23.6)
Perfused vessel density (gut mucosa) (mm/mm^2)	Baseline	15.9 (14.5–22.9)	26.6 (18.7–28.7)
	12 H	16.8 (12.5–20.3)	20.1 (16.0–31.6)
	18 H	20.6 (15.7–26.0)	18.3 (16.5–20.8)
	24 H	17.4 (11.8–27.6)	16.9 (15.1–22.1)
Arterial base excess (mmol/L)	Baseline	4.9 (3.6–5.7)	4.0 (2.8–5.2)
	12 H	0.8 (0.2–1.7)*	0.2 (-0.9–1.5)
	18 H	0.1 (1.6–1.7)*	0.6 (-9.8–0.1)*
	24 H	1.5 (2.7–2.4)*	1.7 (-3.4–1.0)*
Arterial lactate (mmol/L)	Baseline	1.1 (1.0–1.2)	1.1 (1–1.2)
	12 H	1.1 (1.0–1.2)	1 (1–1.1)
	18 H	1.2 (1.1–1.3)	1.3 (1–5.5)
	24 H	1.5 (1.0–2.4)	1.5 (1.3–1.8)
Hemoglobin (g/dL)	Baseline	10.1 (9.7–11.1)	9.9 (8.9–10.5)
	12 H	10.9 (10.5–12.2)*	11.6 (10.4–12)*
	18 H	11.2 (10.8–12.4)*	11.2 (9.6–12.6)*
	24 H	11.9 (10.5–12.4)*	12.7 (12.2–12.8)*

MAP, mean arterial pressure; PAOP, pulmonary artery occlusion pressure; CVP, central venous pressure; DO_2, systemic oxygen delivery; VO_2, systemic oxygen uptake; RBF, renal artery blood flow; CO, cardiac output. #$p < 0.05$ *between SEPSIS and MSC-SEPSIS group;* *$p < 0.05$ *between time-point and baseline.*

sepsis MSC] suggesting a significant population of TCR cells. Importantly, the application of MSCs caused no significant differences when comparing SEPSIS and MSC-SEPSIS groups at each time point (**Figure 6**). Data on full blood count and other inflammatory parameters in sham-operated and septic animals with and without MSC administration are shown in **Supplemental Digital Material** (**Tables 1**, **2**).

In isolated cardiac myocytes, sarcomeric contractions were decreased in septic cells at lower stimulation rates (1, 0.5 Hz). Application of MSC did not exert any significant effect on sarcomeric contraction regardless whether in control (non-septic; not shown) or septic cardiac myocytes (**Figure 7A**). Kinetic parameters of sarcomeric contractions (e.g., time to 50% of peak contraction, time to 50% relaxation) were not affected by sepsis nor by application of MSCs (not shown). Mitochondrial respiration was suppressed in septic hearts and this reduction was mainly due to inhibition of Complex II and IV. Application of MSCs, either in control (not shown) or septic animals, did not affect cardiac mitochondrial respiration (**Figures 7B,C**).

DISCUSSION

The present study was designed to evaluate the short-term safety, tolerability, and efficacy of a single intravenous administration of bone marrow-derived MSCs in a large-animal, peritonitis-induced sepsis model. The model was characterized by the development of the full spectrum of sepsis-induced organ dysfunction with typical hemodynamic, metabolic, and inflammatory host response phenotypes. The main findings indicate that: (1) the application of MSCs to healthy animals was well-tolerated without any measurable acute effects on macro- and microcirculatory hemodynamics, organ, and mitochondrial functions; (2) early treatment with MSCs failed to mitigate the development of sepsis-induced hemodynamic alterations including the progression of sepsis to septic shock; (3) MSCs did not confer any protection against alterations in cellular energy metabolism and multiple organ functions; and (4) treatment failed to counteract a gradual sepsis-driven systemic immune-inflammatory response.

The discrepancy between the salutary effects of MSCs reported in multiple preclinical models (10–15) and the apparent absence of any sign of improvement in multiple biological systems in this model is a striking and key finding of this study, deserving critical discussion. Many emerging treatment strategies shown to be effective in preclinical studies, generally failed to yield beneficial effects in clinical trials. Numerous arguments have been proposed to explain the failure to translate experimental results into effective treatments for human sepsis, including complexity and heterogeneity of sepsis, methodologically inappropriate clinical trials, and clinically irrelevant animal models (4, 16). The latter may prove particularly important in the context of our study. Most notably, all but one study published thus far have involved exclusively rodents, mainly mice (1, 2, 7). The marked difference in the immune-inflammatory response to insults between rodents and humans is well-documented (17). Fundamental differences include, but are not limited to,

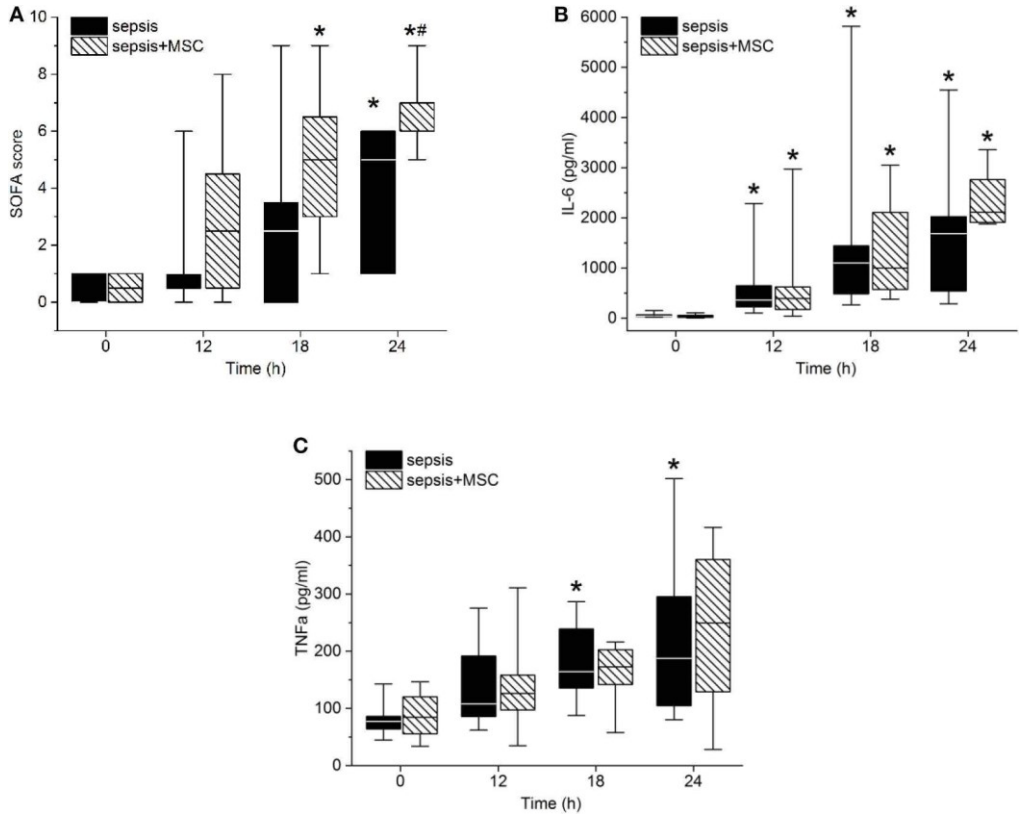

FIGURE 5 | Sepsis-related organ failure assessment (SOFA) score (A), Interleukin-6 (B), and TNF serum levels (C). The (#) stands for statistical signi!cance between the groups ($p < 0.05$). The (*) stands for signi!cant differences in time in particular groups ($p < 0.05$ vs. baseline).

the divergence of the transcriptomic response, the mismatch of temporal response patterns, di[]erences in both innate and adaptive immunity, and/or the homogeneity of highly inbred mouse strains (18). Pigs, on the other hand, show very similar endotoxin sensitivity and tissue antigenicity, similar cardiovascular and renal physiology including hyperdynamic circulation in sepsis and similar temporal response pattern to humans (19). Additional technical advantages are associated with bigger body size that is comparable to humans and allows extensive instrumentation, continuous monitoring, and serial blood sampling. Moreover, frequent use of speci$\square$c pathogen-free (SPF) animals in sepsis research, where alterations of the gut microbiome may markedly alter the animal's immune and in$\square$ammatory functions and susceptibility to infection, may also contribute to the disconnect between animal studies showing promising drug development and failure to translate to humans. In an interesting recent study a more straightforward comparison of response to anesthesia and surgical trauma was made between conventional and SPF rats (20). Comparison between conventional and SPF animals within one species and even strain (Spraque-Dawley) revealed decreased tolerance to anesthesia, hemodynamic instability, aberrant hematology, traumatic bleeding, and reduced physiological reserve in SPF animals. This altered phenotype to the stress of surgical trauma was completely reversed when SPF animals were returned to the original conventional facility. The role of gut microbiota is another aspect worth consideration when discussing factors potentially a[]ecting the host response to infectious stimulus. It has been demonstrated that variations in the gut microbiota of donor mice in$\square$uenced clinical as well as molecular phenotype of sepsis (21). At the time of our study, we did not assess individual microbial composition of porcine feces. However, given the single provider of laboratory animals with identical environmental conditions and comparable individual hemodynamic, metabolic, and in$\square$ammatory responses to feces, we hypothesize that the putative role of inter-individual variations in fecal microbiota was rather limited in this experiment. Taken together, the genetic and physiological proximity of pigs and humans makes this species an excellent biomodel for translational research, and utilization of domestic (farm) pigs exposed to similar environmental pathogens as humans is clearly superior to mouse models, in which the SPF handling probably limits the clinical translatability even more fundamentally (22, 23).

Our results are also in sharp contrast with data from Laroye et al., who reported less profound alterations in systemic hemodynamics and, therefore, longer time to death in septic pigs treated with human umbilical cord-derived MSCs (7). Surprisingly, the hemodynamic improvement observed in the above study occurred despite the lack of clear evidence for signi$\square$cant e[]ects of MSCs on in$\square$ammation, bacterial clearance,

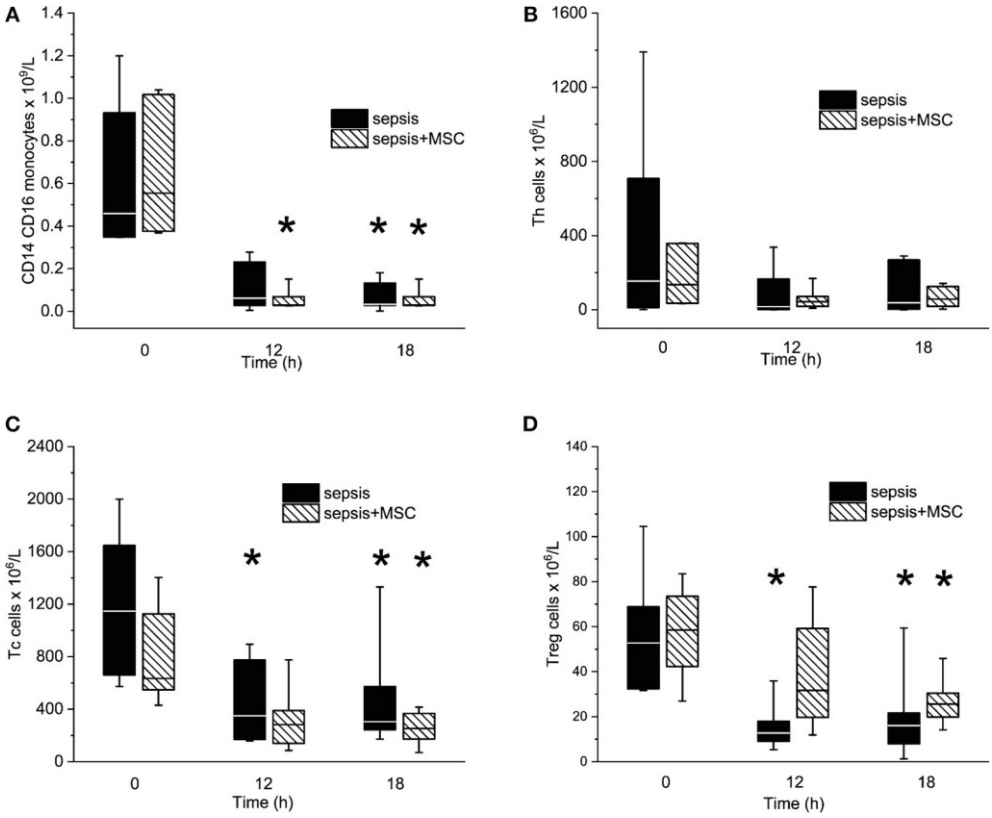

FIGURE 6 | The absolute numbers of CD14/CD16^{pos} monocytes (A), T helper lymphocytes (B), Tc CD8 T lymphocytes (C), and T regulatory lymphocytes (D). The (*) stands for signi!cant difference between particular time point and baseline only ($p < 0.05$).

and a rather transient alleviation of lung and kidney function. Although the dose and timing of interventions with MSCs were comparable to those reported here, the two studies di[]ered with respect to the source of MSCs. As the secretome of umbilical cord-derived MSCs used by Laroye et al. might di[]er from bone marrow-derived MSCs, it is possible that MSCs from di[]erent sources possess di[]erent immunomodulatory capacity (24, 25). Bone marrow-derived MSCs have not only been proven to be e[]ective in several small-animal sepsis studies (10, 24, 26), but they are currently the preferred source of MSCs in clinical settings and the only one with the ability to restore the original niche (27). Nevertheless, in a mouse model of endotoxin-induced sepsis, MSCs ameliorated sepsis-associated organ injury and mortality in spite of di[]erent MSC sources (26). Furthermore, the infectious burden and severity of sepsis were three times higher in Laroye's experiment compared to our study and the time course of pro-in[illegible]ammatory cytokines, in particular TNF- , markedly di[]ered from our model, thus suggesting a di[]erent in[illegible]ammatory environment. Whether these di[]erences may have accounted for such discrepancy in results remains speculative.

Understanding existing methodological limitations and obstacles is essential for further translational research. Even though they were used successfully in previous experiments (28), we investigated the e[]ects of one dose and one source of MSCs. Furthermore, MSCs were administered at a single time point. It would be of interest to evaluate the e[illegible]cacy of MSCs obtained from di[]erent tissue sources, given in a dose-response fashion, and administered at di[]erent time points within the same experimental setting. The timing may be of critical importance for the interpretation of our results, because in the vast majority of positive preclinical studies, MSCs were administered very early, i.e., within 4 h after sepsis induction (2). No study has tested the application of MSCs beyond a 6-h time window (2). Indeed, the potential role of timing of the intervention has recently been demonstrated in a human endotoxemia model (29). In that study, intravenous infusion of allogenic human adipose MSCs exerted mixed time-dependent pro-in[illegible]ammatory or anti-in[illegible]ammatory and pro-coagulant e[]ects (28). This suggested considerable biological complexity of MSCs and, possibly, a relatively narrow time frame for the treatment of early sepsis. Another relevant issue is the role of antibiotics in potentiating the action of MSCs. In a mouse model of sepsis induced by cecal ligation and puncture, combined treatment with MSCs and antibiotics greatly improved sepsis-associated symptoms and survival, indicating some synergistic e[]ect (30). In our study, antibiotic therapy was not used. Our model was designed to create hyperdynamic sepsis, with increasing severity over time. Antibiotic therapy was expected to blunt the host response, thereby attenuating the development and full manifestation of a true clinical septic response during 24 h,

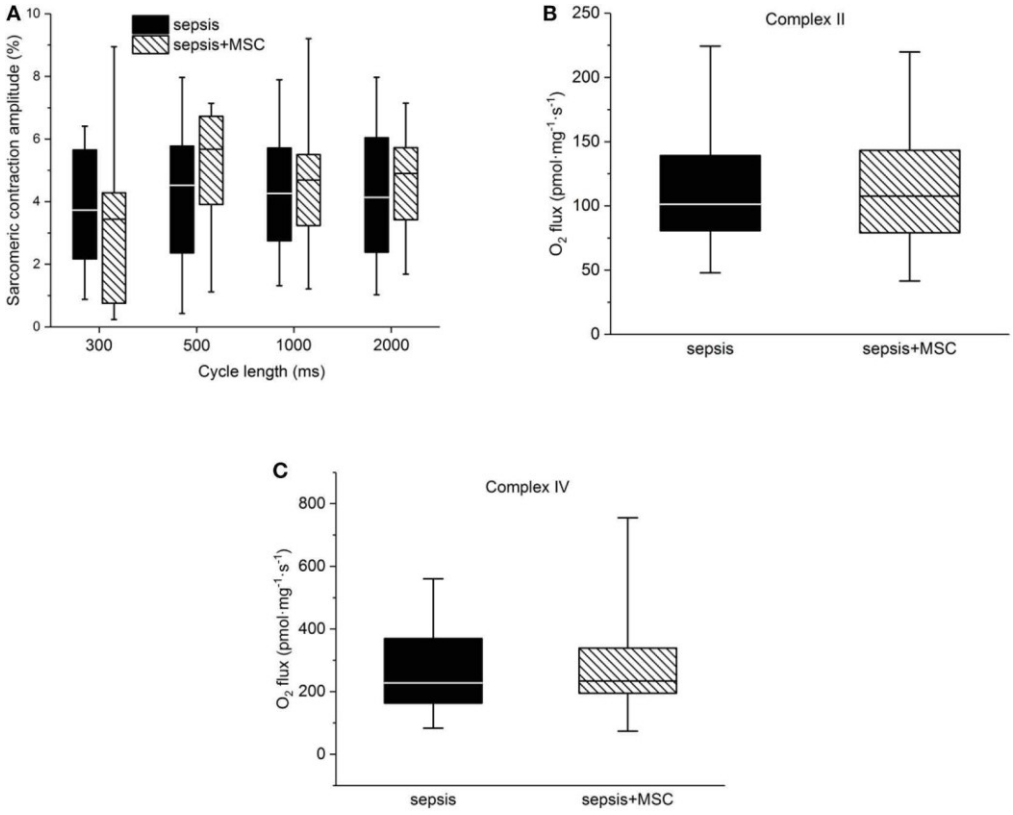

FIGURE 7 | Cellular myocardial depression. (A) Relative sarcomeric contraction amplitudes in cardiac myocytes from septic animals with or without application of MSC. (B) Mitochondrial respiration. Complex II-dependent oxygen consumption in septic myocardium without or with application of MSC. (C) Mitochondrial respiration. Complex IV-dependent oxygen consumption in septic myocardium without or with application of MSC.

which is what we instead sought to elicit in our experiment. Nevertheless, the interactions between antibiotic therapy and MSCs represent another important question warranting further investigation. We cannot exclude that the protective e[]ect of MSCs might have manifested through other markers/pathways not monitored in our study. However, given that we assessed several clinically relevant and mutually independent biological targets, including macro- and microcirculatory perfusion, multiple organ functions, mitochondrial energy metabolism, systemic immune-in[ammatory response, and oxidative stress, it seems unlikely that we would have missed major treatment e[]ects.

The manufacturing of MSCs for this study was consistent with procedures applied elsewhere using fetal bovine serum for MSC propagation, trypsin for MSC harvesting, and a single freeze/thaw cycle for MSCs cryopreservation (10, 11, 15). Transplanted MSCs conformed to the criteria of the International Society for Cellular Therapy (31) and were kept for the minimum number of passages (up to four) to maintain their stem cell phenotype and avoid senescence (32). Paradoxically, the inconsistency in MSCs potency could lie in their sensitivity to a cytokine environment (33). As each pathology and even patient serum is accompanied by speci[c qualitative and quantitative cytokine composition, the immunomodulatory function of MSCs can be highly dependent on a particular patient's response to a speci[c pathology or treatment.

Another issue with possible role in MSCs potency is their matching to the recipient. There is the lack of studies comparing e[]ects of matched vs. mismatched MSCs in the large animal model of sepsis. However, the clinical study of the team of Garcia-Sancho (34) showed that better HLA matching of donor MSCs with recipients did not enhance the e[cacy of MSCs therapy in osteoarthritis and degenerative disc disease. Furthermore, there are several completed clinical trials applying allogenic MSC without any analysis of donor MSCs and recipient matching (5, 35–37) indicating that the donor MSCs and recipient matching may not play the role in MSCs potency. This direction is further supported by tendencies to apply pooled MSCs batches as better available and reproducible source of MSCs (38). However, upon the lack of available data comparing matched vs. mismatched MSCs therapy in sepsis relevant animal models we can only speculate about the role of this issue in MSCs e[cacy in general.

Despite these limitations, our study bene[ts from a number of strengths and important [ndings. The history of sepsis research has repeatedly shown that no new therapeutic approach that was successfully tested in preclinical models was e[ective in clinical practice (39). Our model replicates many of the biological features intrinsic to human septic shock and

integration of standard day-to-day care resuscitative measures makes it an appealing sepsis model in translational research. The International Society for Stem Cell Research has recently released guidelines for clinical translation stating strongly that large-animal models should be used for stem cell research and that any clinical trial should be preceded by compelling preclinical evidence obtained from these models (40). The clinical application of MSCs that we are witnessing in the fi eld of orthopedics or neurology, and which is based on minimal evidence of bene fi t and safety, represents a path that critical care medicine should avoid (1). Generally homogenous and encouraging results attained by current preclinical testing cannot be considered as su fi cient arguments for launching clinical trials, in part, due to a considerable risk that the e[]ect of MSCs is overstated given that a number of studies with negative results have not been published.

CONCLUSION

In this large, clinically relevant animal peritonitis-induced sepsis model, MSCs were not capable of reversing any of the sepsis-induced disturbances in multiple biological, organ, and cellular systems. Collectively, our study cautions against the use of MSCs in a complex disease such as sepsis, as our understanding of the role MSCs play in it is still incomplete and unknown factors may in fl uence the outcome.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee for Experiments on Animals of the Faculty of Medicine, Charles University, in Pilsen and by the Ministry of Education, Youth and Sports of the Czech Republic (protocol no. MSMT-20064/2015-3).

AUTHOR CONTRIBUTIONS

JH, LN, VM, VT, and JB performed animal experiments. MM and MS conceived and designed the study. MM, MS, LV, and JH performed data analysis. MM and JH drafted the manuscript. LV and MD performed MSC processing and fl ow cytometry measurements. JK and MK performed mitochondrial function measurement and data analysis. MS and DJ performed cardiac myocytes measurements and data analysis. All authors contributed to data interpretation, editing of the manuscript, and read and approved the fi nal manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00126/full#supplementary-material

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Cellular Mechanisms of Myocardial Depression in Porcine Septic Shock

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The complex pathogenesis of sepsis and septic shock involves myocardial depression, the pathophysiology of which, however, remains unclear. In this study, cellular mechanisms of myocardial depression were addressed in a clinically relevant, large animal (porcine) model of sepsis and septic shock. Sepsis was induced by fecal peritonitis in eight anesthetized, mechanically ventilated, and instrumented pigs of both sexes and continued for 24 h. In eight control pigs, an identical experiment but without sepsis induction was performed. *In vitro* analysis of cardiac function included measurements of action potentials and contractions in the right ventricle trabeculae, measurements of sarcomeric contractions, calcium transients and calcium current in isolated cardiac myocytes, and analysis of mitochondrial respiration by ultrasensitive oxygraphy. Increased values of modi ed sequential organ failure assessment score and serum lactate levels documented the development of sepsis/septic shock, accompanied by hyperdynamic circulation with high heart rate, increased cardiac output, peripheral vasodilation, and decreased stroke volume. In septic trabeculae, action potential duration was shortened and contraction force reduced. In septic cardiac myocytes, sarcomeric contractions, calcium transients, and L-type calcium current were all suppressed. Similar relaxation trajectory of the intracellular calcium-cell length phase-plane diagram indicated unchanged calcium responsiveness of myo laments. Mitochondrial respiration was diminished through inhibition of Complex II and Complex IV. Defective calcium handling with reduced calcium current and transients, together with inhibition of mitochondrial respiration, appears to represent the dominant cellular mechanisms of myocardial depression in porcine septic shock.

Keywords: sepsis, pig, myocardial depression, calcium, mitochondria

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INTRODUCTION

Sepsis represents a well-recognized worldwide health problem. Based on the meta-analysis of studies from developed high-income countries, global annual estimates were 31.5 million sepsis and 19.4 million severe sepsis cases, with potentially 5.3 million deaths, in the hospital setting (Fleischmann et al., 2016). Sepsis plays a prominent role in hospital mortality: in two large

complementary inpatient cohorts (total of 157,518 deaths/7,038,449 admissions) sepsis was found to contribute to 1 in every 2 to 3 deaths (Liu et al., 2014).

Sepsis is de[ned as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016). A common manifestation of sepsis/septic shock is myocardial depression with reversible biventricular dilatation and depressed ejection fraction (Antonucci et al., 2014). The precise mechanistic link between infection, sepsis, and myocardial depression remains unclear, although a number of possible pathways have been suggested (Merx and Weber, 2007; Sato and Nasu, 2015). An early theory of global myocardial ischemia due to inadequate coronary blood [ow in sepsis was disproved by [ndings of high coronary blood [ow and diminished coronary arteriovenous oxygen di[glerence in septic patients (Cunnion et al., 1986). Nowadays, there is a general consensus on the mechanism of a circulating depressant substance, as originally demonstrated by Parrillo et al. (1985). The exact nature of the circulating depressant substance, however, has not been su[:iciently clari[ed yet. The list of possible candidates includes various cytokines, endotoxins, prostanoids, and nitric oxide (Merx and Weber, 2007; Sato and Nasu, 2015).

Similarly, the downstream cellular pathophysiology of myocardial depression is still obscure, although in experimental models, a number of contributing mechanisms have been reported. The cardiac cellular mechanisms include a reduction of L-type calcium current (I_{CaL}) (Lew et al., 1996; Zhong et al., 1997; Stengl et al., 2010), altered calcium transients (Ren et al., 2002), increased calcium leakage from the sarcoplasmic reticulum (Zhu et al., 2005), impaired sarcolemmal diastolic calcium extrusion pathways (Wagner et al., 2015), oxidation and subsequent activation of calcium and calmodulin-dependent protein kinase with phosphorylation of the ryanodine receptor (Sepúlveda et al., 2017), altered phosphorylation and calcium sensitivity of cardiac myo[brillar proteins (Wu et al., 2001), and/or mitochondrial dysfunction (Levy et al., 2004; Watts et al., 2004).

Most data on the intrinsic cellular mechanisms of myocardial depression, however, were obtained in small animal (rodent) experimental models with limited clinical relevance and translatory potential (Poli-de-Figueiredo et al., 2008; Dyson and Singer, 2009). To overcome this limitation, cellular mechanisms of myocardial depression were examined in a clinically relevant porcine model of peritonitis-induced progressive septic shock, which, in contrast to rodent hypodynamic endotoxic shock, closely mimics human sepsis (hyperdynamic circulation with low systemic vascular resistance and multiple organ dysfunction). The myocardial functions were examined on several levels of biological complexity, from the *in vivo* experiment similar to the clinical scenario down to experiments in isolated cells and organelles, with special emphasis on calcium homeostasis and mitochondrial function.

MATERIALS AND METHODS

Animal handling was in accordance with the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scienti[c Purposes (86/609/EU). The experiments were approved by the Committee for Experiments on Animals of the Charles University Faculty of Medicine in Pilsen and by the Ministry of Education, Youth and Sports of the Czech Republic (Protocol No. MSMT-24725/2014-05). All experiments were performed in the animal research laboratory at the Faculty of Medicine in Pilsen. Sixteen domestic pigs of both sexes and of similar weight (43.9 5.8 kg) were used for experiments. Sepsis was induced by fecal peritonitis in eight pigs (seven boars, one sow) while control sham experiments (analogous procedure but without sepsis induction) were performed in another eight pigs (four boars, four sows).

Anesthesia and Instrumentation

Anesthesia and instrumentation protocols were similar to those previously described (Jarkovska et al., 2016). Anesthesia was induced with intramuscular (IM) tiletamine (2.2 mg/kg), zolazepam (2.2 mg/kg), and xylazine (2.2 mg/kg), together with intravenous (IV) propofol 2% (1–2 mg/kg) and maintained with continuous IV propofol (1–4 mg/kg/h) and fentanyl (5–10 g/kg/h). Animals were mechanically ventilated (FiO_2 0.3, PEEP 8 cm H_2O, tidal volume 10 ml/kg, respiratory rate adjusted to maintain end/tidal pCO_2 between 4 and 5 kPa), and muscle paralysis was achieved with IV rocuronium (4 mg for induction, 0.2–0.4 mg/kg/h for maintenance). Ringerfundin solution (B. Braun Melsungen AG, Melsungen, Germany) was infused as maintenance [uid (7 ml/kg/h) and normoglycemia (til blood glucose level 4.5–7 mmol/L) was maintained using 10% glucose infusion (1–4 ml/kg/h).

All pigs were instrumented with a femoral artery catheter, triple lumen central venous catheter, and pulmonary artery catheter. Silicone drains directed into the anatomical spaces of Morison and Douglas were used for fecal inoculation.

Experimental Protocol

Experimental protocols were identical to those previously described (Jarkovska et al., 2016). Peritonitis was induced by inoculating 1 g/kg of autologous feces (cultivated for 10 h in 100 ml isotonic saline at 37 C) into the abdominal cavity. In addition to continuous crystalloid infusion, [id boluses (10 ml/kg of Ringerfundin) were administered to maintain cardiac output and mean arterial pressure (MAP) in a goal-directed fashion. Continuous IV norepinephrine was administered if MAP fell below 65 mmHg despite [id administration and titrated to maintain MAP above 70 mmHg. In total, the experiments lasted 34 h (4 h for surgical instrumentation, 6 h of recovery, and 24 h after induction of peritonitis). At the end of the experiment, the animals were euthanized by anesthetic overdose and excision of the heart.

Measurements

Systemic and pulmonary hemodynamics were measured and electrocardiography (lead II) was performed as described previously (Stengl et al., 2010; Jarkovska et al., 2016). The modi[ed sequential organ failure assessment (SOFA) score was determined according to the Third International Consensus De[nitions for Sepsis and Septic Shock (Singer et al., 2016)

and modified by exclusion of the Glasgow Coma Scale-based neurologie component.

Action potentials and isometrie contractions in the right ventricle trabeculae were recorded as described previously(Stengl et al., 2008, 2010, 2013). Action potentials were recorded with high-resistance(> 20 MO) glass microelectrodes filled with 3 M KCI at various stimulation frequencies (3, 2, 1, O.S Hz), and simultaneously, isometrie contractions were recorded using an isometrie force transducer (F30, HugoSachs, March-Hugstetten, Germany). Aliaction potential [APD90, action potential duration at the 90% Jeve ! of repolarization, action potential amplitude (APA), resting membrane potential (RMP)) and contraction (contraction force, time from resting tension to the peak of contraction, time to 90% relaxation) parameters were measured in Sbeatsandaveraged, and the meanvalues were usedfor further analyses and comparisons.

Cardiac myocytes were isolated from the left ventricle by enzymatie dissociation (collagenase A from Sigma-Aldrieh, St. Louis, MO, United States) as previously reported (Stengl et al., 2010). lcaL was measured using the whole-cell configuration of the patch-clamp technique at 36°C (Stenglet al., 2010).

Sarcomerie contractions and calcium transients of isolated cardiac myocytes were measured with Ionoptix HyperSwitch Myocyte Calcium and Contractility System (IonOptix LLC, Westwood, CA, United States), with the Sarclen sarcomere length acquisition module. Cells were loaded with Fura-2 (Molecular Probes, Invitrogen, Waltham, MA, United States). For stock solution Fura-2-am powder was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrieh, St. Louis, MO, United States) to reach a final concentration of 1 mmol/L. Cells were incubated for 20 min in norma!calcium Tyrodesolution with 2 µmol/L Fura-2-amand then repeatedly washed with norma! calcium Tyrode solution. Measurements were performed in norma! Tyrode solution at 37 ± 0.5°C. Cells were stimulated with a field stimulator (MyoPacer Field Stimulator, IonOptix LLC, Westwood, CA, United States) at frequencies of3,2,1, O.S Hz . For ofiline analysis ofsarcomerie contractions and calcium transients, the IonWizard 6.5 software (IonOptix LLC, Westwood, CA, United States) was used.

Cardiac mitochondrial function was assessed using high-resolution respirometry (oxygraph Oroboros; Oroboros Instruments, Innsbruck, Austria). Samples of left ventrieular myocardium (1.5- 2.0 mg) were permeabilized by saponin in BIOPS solution (Cantó and Garcia-Roves, 2015). The fibers were then washed with respiration medium containing catalase and placed into oxygraph chambers with MiR06 medium equilibrated with air. Mitochondrial oxygen consumption was measured at 37°C after raising oxygen concentration to 400- 500 µ mol/L by titration of H20 2 (200 mmol/L). ln the titration protocol, different substrates and inhibitors of the mitochondrial respiratory system were sequentially added into the chambers to determine various respiratory states; non-phosphorylating LEAK state (L, oxygen consumption needed for electron transport compensating for proton leak across the inner mitochondrial membrane, induced by the addition of substrates providing electrons to Complex I - malate, 2 mmol/L, glutamate, 10 mmol/L, and pyruvate, S mmol/L), OXPHOS I (active phosphorylating respiration induced by S mmol/L ADP), OXPHOS 1c (the degree of cell membrane permeabilization verified with cytochrome c 10 µ mol/L), OXPHOS I+II (mitochondrial respiration increased by succinate, Complex II substrate, 10 mmol/L), OXPHOS II (reflecting activity of Complex II, induced by inhibition of Complex I by rotenone, O.S µ mol/L), ROX (residua! oxygen consumption after complex III inhibition by antimycin A, 2.5 µmol/L), and Complex IV activity (respirometrie assay for cytochrome c oxidase activity by simultaneous injection of *N,N,N′,N′*-tetramethyl-p-phenylenediamine dihydrochloride, TMPD, O.S mmol/L, and ascorbate, 2 mmol/L).

The oxygen consumption was analyzed online by DatLab software (Oroboros Instruments, Innsbruck, Austria) as the negative time derivative of oxygen concentration in the chamber, expressed in pmol 02/(s·mg tissue wet weight), and corrected to ROX.

Citrate synthase activity serving as a marker of mitochondrial content was measured in all samples taken from the oxygraph chambers. The assay medium was mixed with homogenized chamber content and citrate synthase activity was measured spectrophotometrieallyat 412 nmand 30°C, and expressed in IU per gtissue weight (Kuznetsovet al., 2002).

Solutions and Drugs

The composition of the Tyrode solution was as follows (in mmol/L): NaCl 137, KCI 4.5, MgC!i 1, CaC!i 2, glucose 10, HEPES S; pH was adjusted to 7.4 with NaOH. The patch-clamp pipette solution contained: cesium glutamate 125, tetraethylammonium chloride 25, MgC!i 1, Na2ATP S, EGTA 1, HEPES S; pH adjusted to 7.2 with CsOH. BIOPS solution was composed of CaK2EGTA 2.77, K2EGTA 7.23, Na2ATP 5.77, MgCli·6H20 6.56, taurine 20, Na2 Phosphocreatine 15, imidazole 20, dithiothreitol O.S, and MES hydrate 50, with pH adjusted to 7.1. (Pesta and Gnaiger, 2012). MiR06 respiration medium contained EGTA 0.S, MgC!i·6H20 3, K-lactobion ate 60, taurine 20, KH2P0 4 10, HEPES20, sucrose llO, fatty acid free bovine serum albumin 1 g/L, and catalase 280 U/mL at pH 7.0 (Gnaigeret al., 2000). The composition ofthe assay medium for determination of citrate synthase activity was 5,5-dithio -bis-(2-nitrobenzoie) acid 0.1, oxaloacetate O.S, acetyl coenzyme A 0.31, triethanolamine hydrochloride S, Tris-HCI 100, and S µmo/L EDTA, Triton -X 0.25%, pH adjusted to 8.1). Ali chemicals were from Sigma-Aldrieh(St. Louis, MO, United States).

Statistical Analysis

Results are presented as means ± SD. After testing for normality of distribution (Shapiro- Wilk test), statistical comparisons were made with the two-way mixed-design ANOVA (one repeated-measures factor for analysis of the progression of parameter in time and one between-groups factor for comparison between control and septic groups, *in vivo* data) followed by *post hoc* Tukey test or by unpaired t-test (control vs. septic group comparison of *in vitro* data). The analysis was performed using the software Origin 2017 (Orig inLab,Corp., Northampton, MA, United States) or STATISTICA Cz 8 (StatSoft, Inc., Prague, Czechia). Values of $p < O.OS$ were considered significant.

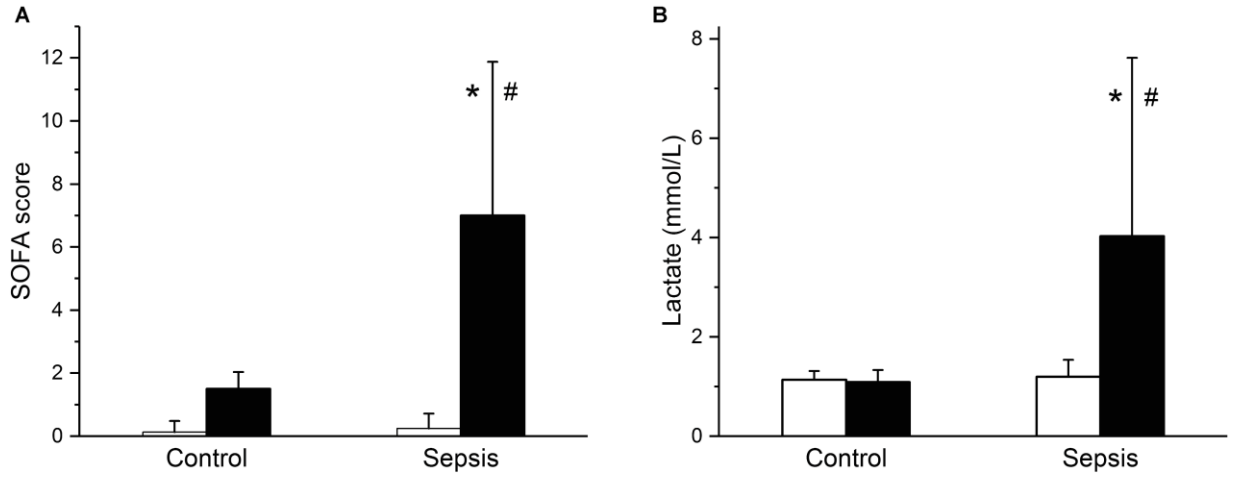

FIGURE 1 | Modi ed sequential organ failure assessment (SOFA) score and serum lactate. (A) Modi ed SOFA score in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. (B) Serum lactate levels in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. Signi cantly different from start of the experiment, $p < 0.05$; # signi cantly different from control, $p < 0.05$.

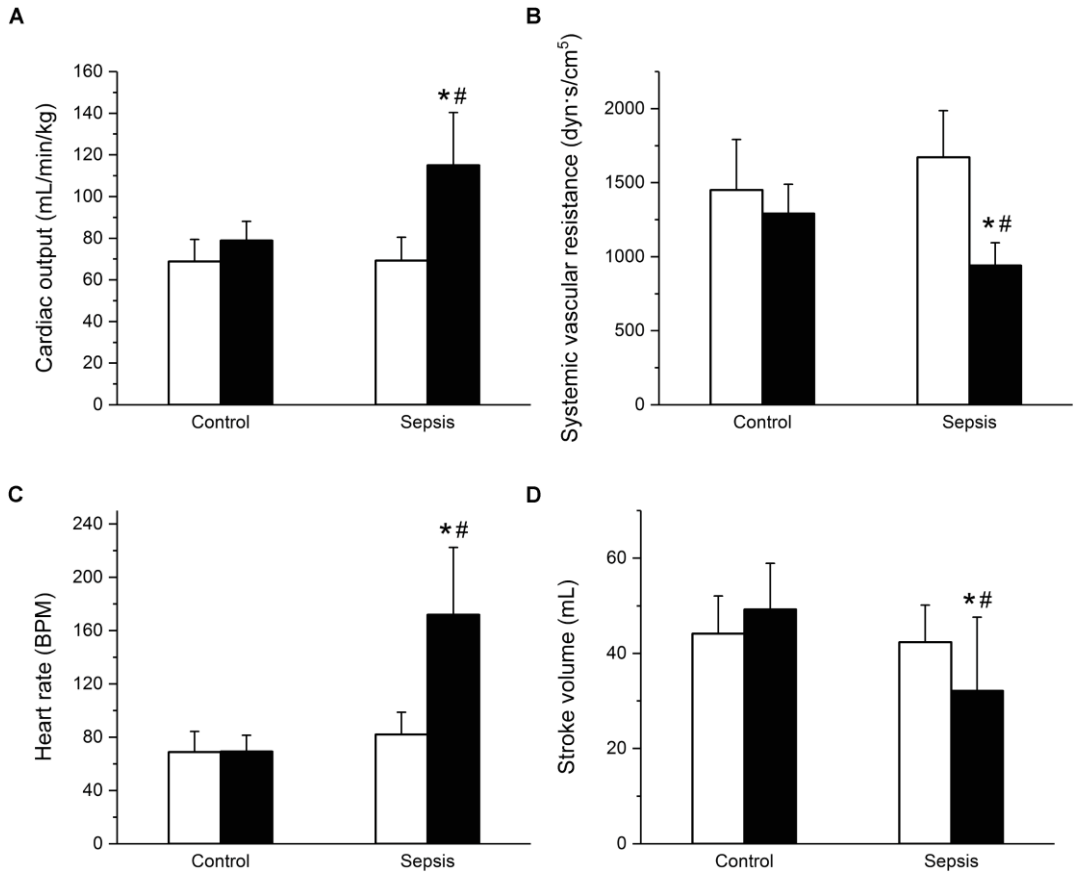

FIGURE 2 | Hyperdynamic circulation in sepsis. (A) Cardiac output in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. (B) Systemic vascular resistance in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. (C) Heart rate in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. (D) Stroke volume in control ($n = 8$) and septic ($n = 8$) animals. Empty columns, start of experiment; lled columns, end of experiment. Signi cantly different from start of the experiment, $p < 0.05$; # signi cantly different from control, $p < 0.05$.

RESULTS

In the septic group, development of sepsis and septic shock with organ dysfunction was manifested by a signi[cant increase of the total modi[ed SOFA score (**Figure 1A**) and of serum lactate levels (**Figure 1B**). Out of eight animals in this group, septic shock requiring vasopressors and with lactate levels over 2 mmol/L developed in six animals; in two animals, vasopressor administration was not required, but the criteria for sepsis was ful[lled based on their SOFA scores. Concurrently, the septic animals developed hyperdynamic circulation with increased cardiac output and peripheral vasodilation (**Figures 2A,B**). The increase in cardiac output was predominantly due to elevated heart rate (**Figure 2C**), as the stroke volume was reduced (**Figure 2D**). The control animals did not show any signs of systemic in[ammatory reaction, their SOFA scores remained normal (**Figure 1A**), their lactate levels remained low throughout the run of the experiment, and none of them needed vasopressor support. Their global hemodynamic parameters remained unchanged compared to the baseline (**Figures 2A–D**).

In cardiac trabeculae, sepsis induced a shortening of action potential duration (**Figures 3A,B**) at lower stimulation rates (1, 0.5 Hz) and reduction of contraction force (**Figures 3C,D**). Values of APA and RMP were similar in control and septic preparations (e.g., at 1 Hz APA of 101 5 mV in control vs. of 100 12 mV in sepsis; RMP of 71 7 mV in control vs. 70 7 mV in sepsis). Kinetics of trabecular contraction (time to peak, TTP; time to 90% relaxation, R90) were not a[glected by sepsis (e.g., at 1 Hz TTP of 184 53 ms in control vs. 171 57 ms in sepsis; R90 of 243 80 ms in control vs. 240 61 ms in sepsis).

In isolated myocytes, sarcomeric contractions (**Figures 4A,B**) and calcium transients (**Figures 4C,D**) were reduced in septic

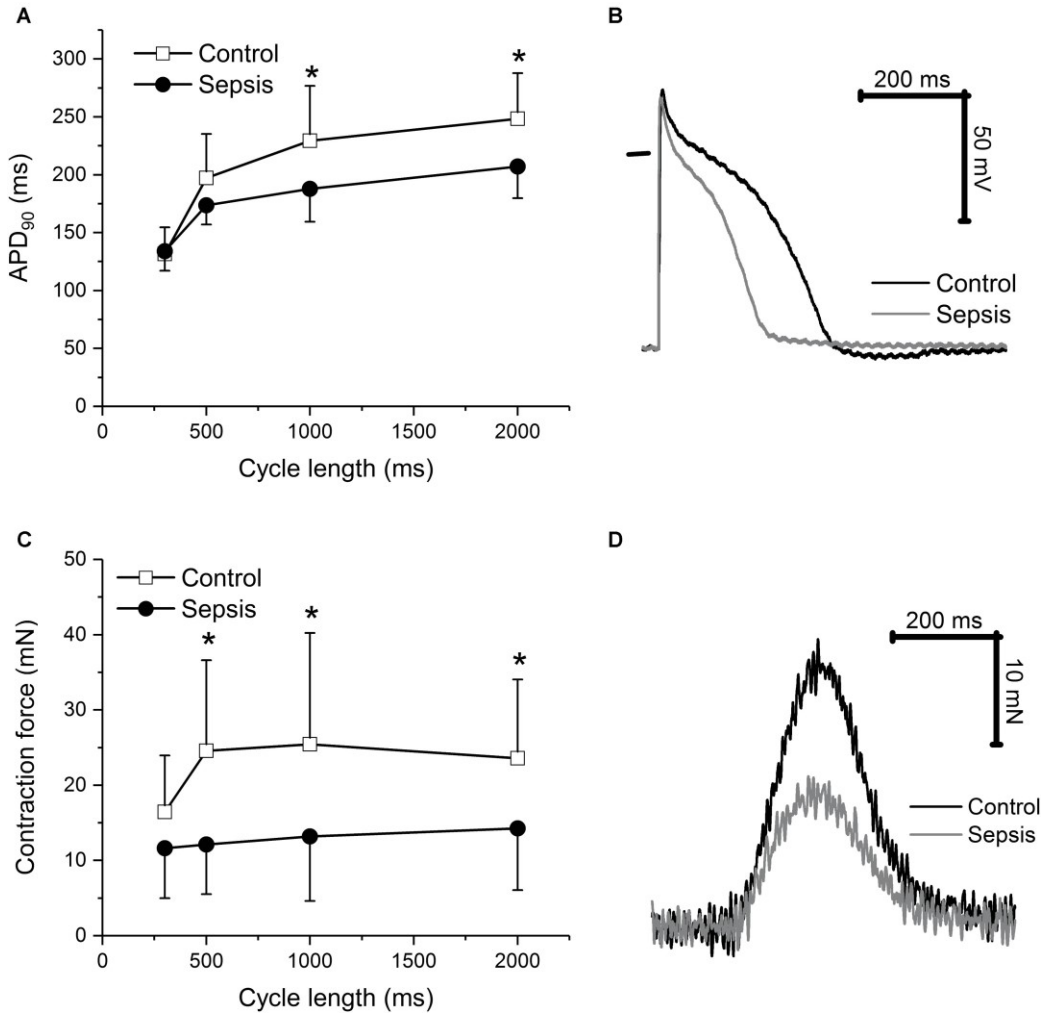

FIGURE 3 | Action potential and contraction in cardiac trabeculae. (A) Action potential duration at 90% repolarization in cardiac trabeculae from control ($n = 8$) and septic ($n = 8$) animals. Empty squares, control; lled circles, sepsis. (B) Representative action potentials of cardiac trabeculae from control and septic animals. Stimulation frequency of 1 Hz. Black line, control; gray line, sepsis. (C) Contraction force in cardiac trabeculae from control ($n = 8$) and septic ($n = 8$) animals. Empty squares, control; lled circles, sepsis. (D) Representative contractions of cardiac trabeculae from control and septic animals. Stimulation frequency of 1 Hz. Black line, control; gray line, sepsis. Signi cantly different from control, $p < 0.05$.

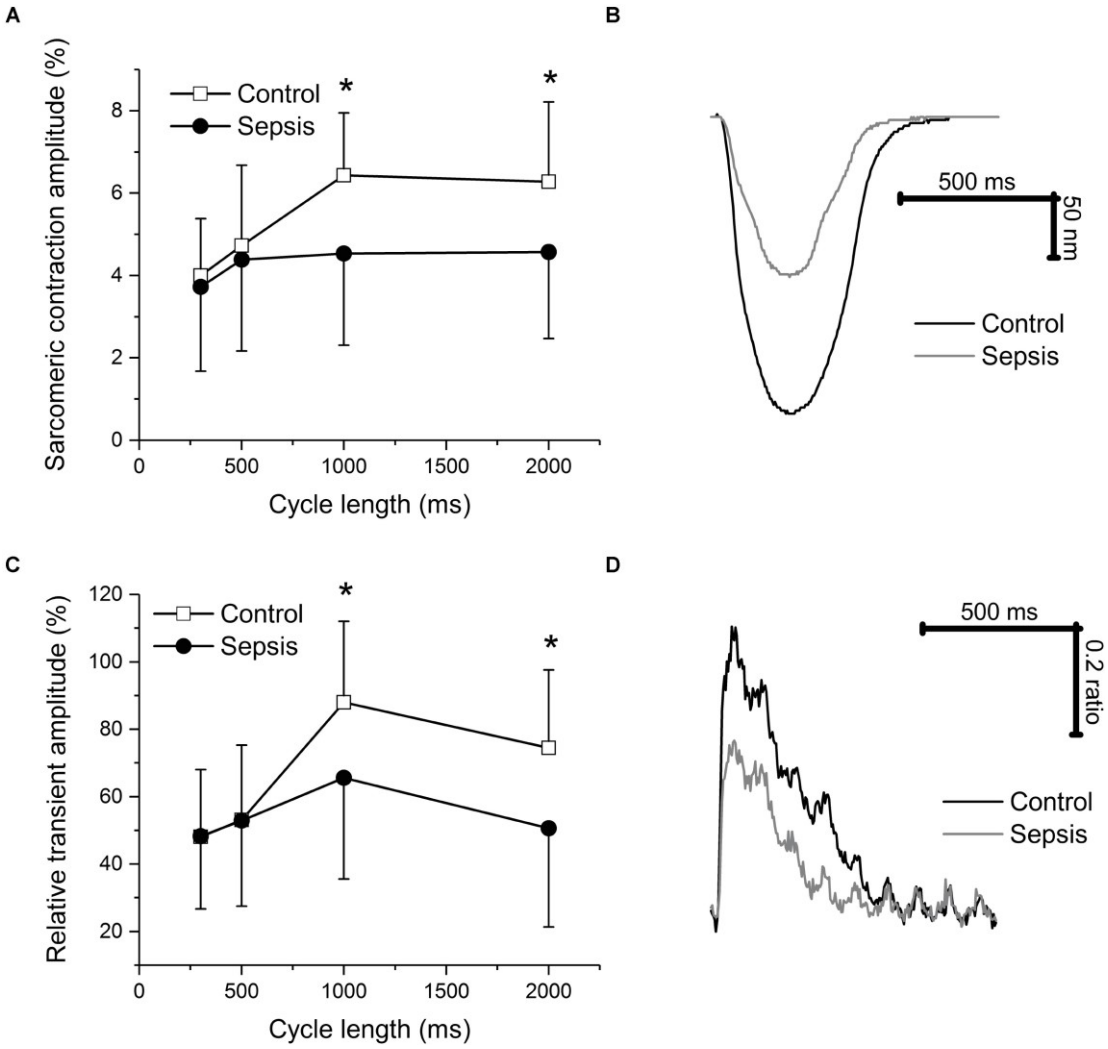

FIGURE 4 | Sarcomeric contraction and calcium transients in cardiac myocytes. (A) Relative sarcomeric contraction amplitudes in cardiac myocytes from control and septic animals. Empty squares, control ($n = 14$); lled circles, sepsis ($n = 21$). (B) Representative sarcomeric contractions of cardiac myocytes from control and septic animals. Stimulation frequency of 1 Hz. Black line, control; gray line, sepsis. (C) Relative calcium transient amplitudes in cardiac myocytes from control and septic animals. Empty squares, control ($n = 14$); lled circles, sepsis ($n = 21$). (D) Representative calcium transients of cardiac myocytes from control and septic animals. Stimulation frequency of 1 Hz. Black line, control; gray line, sepsis. Signi cantly different from control, $p < 0.05$.

cells at lower stimulation rates (1, 0.5 Hz). The resting intracellular calcium concentrations were not a[glected by sepsis at any stimulation frequency (e.g., at 1 Hz ratio of 0.588 0.084 in control vs. 0.623 0.115 in septic myocytes). Kinetic parameters of both sarcomeric contractions and calcium transients (time to 50% peak, TP50; time to 50% relaxation, TR50) were not a[glected by sepsis (e.g., for sarcomeric contractions at 1 Hz, TP50 of 78 27 ms in control vs. 84 29 ms in septic myocytes; TR50 of 365 117 ms in control vs. 391 104 ms in septic myocytes; for calcium transients at 1 Hz, TP50 of 12 3 ms in control vs. 12 3 ms in septic myocytes; TR50 of 316 90 ms in control vs. 322 110 ms in septic myocytes). I_{CaL} was decreased in septic cardiac myocytes at membrane potentials between 20 and 40 mV (**Figures 5A,B**).

Since sarcomeric contractions and calcium transients were recorded simultaneously in each cardiac myocyte, phase-plane trajectories of sarcomeric length-intracellular Ca^2 relationship were constructed (**Figure 6A**) and analyzed. The sarcomeric length-intracellular Ca^2 trajectory during the relaxation phase of the twitch contraction was similar in control and septic myocytes (**Figure 6B**). Linear fitting of these relaxation phase trajectories revealed similar slopes (**Figure 6C**) for control and septic myocytes.

Mitochondrial respiration was suppressed in septic hearts (**Figure 7**). Oxygen consumption in the LEAK state was decreased (**Figure 7C**). Mitochondrial respiration in the presence of ADP and Complex I and II substrates (OXPHOS I II) was reduced by sepsis, and this reduction was mainly due to inhibition of Complex II (OXPHOS II), while Complex I activity was

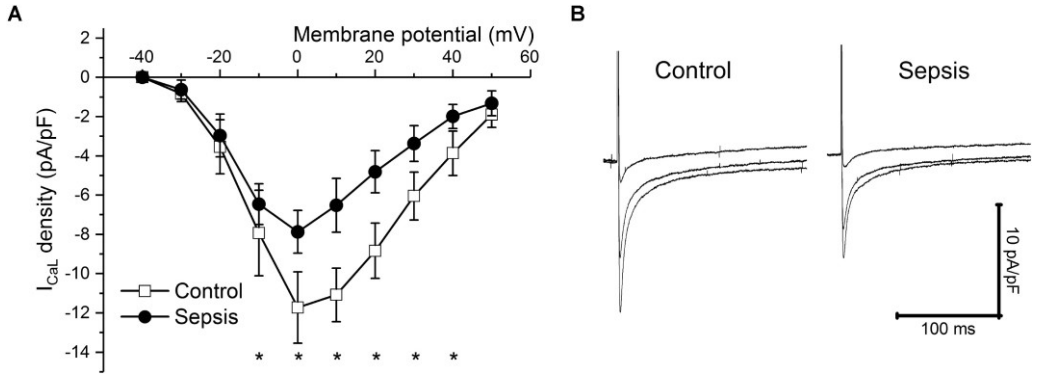

FIGURE 5 | I_{CaL} in sepsis. (A) I_{CaL} density at various membrane potentials (–40 to +50 mV) in control (empty squares, n = 10) and septic (lled circles, n = 10) cardiac myocytes. Signi cantly different from control, $p < 0.05$. (B) Representative I_{CaL} traces at membrane potentials of 0, +20, and +40 mV of cardiac myocytes from control and septic animals. Left panel, control; right panel, sepsis.

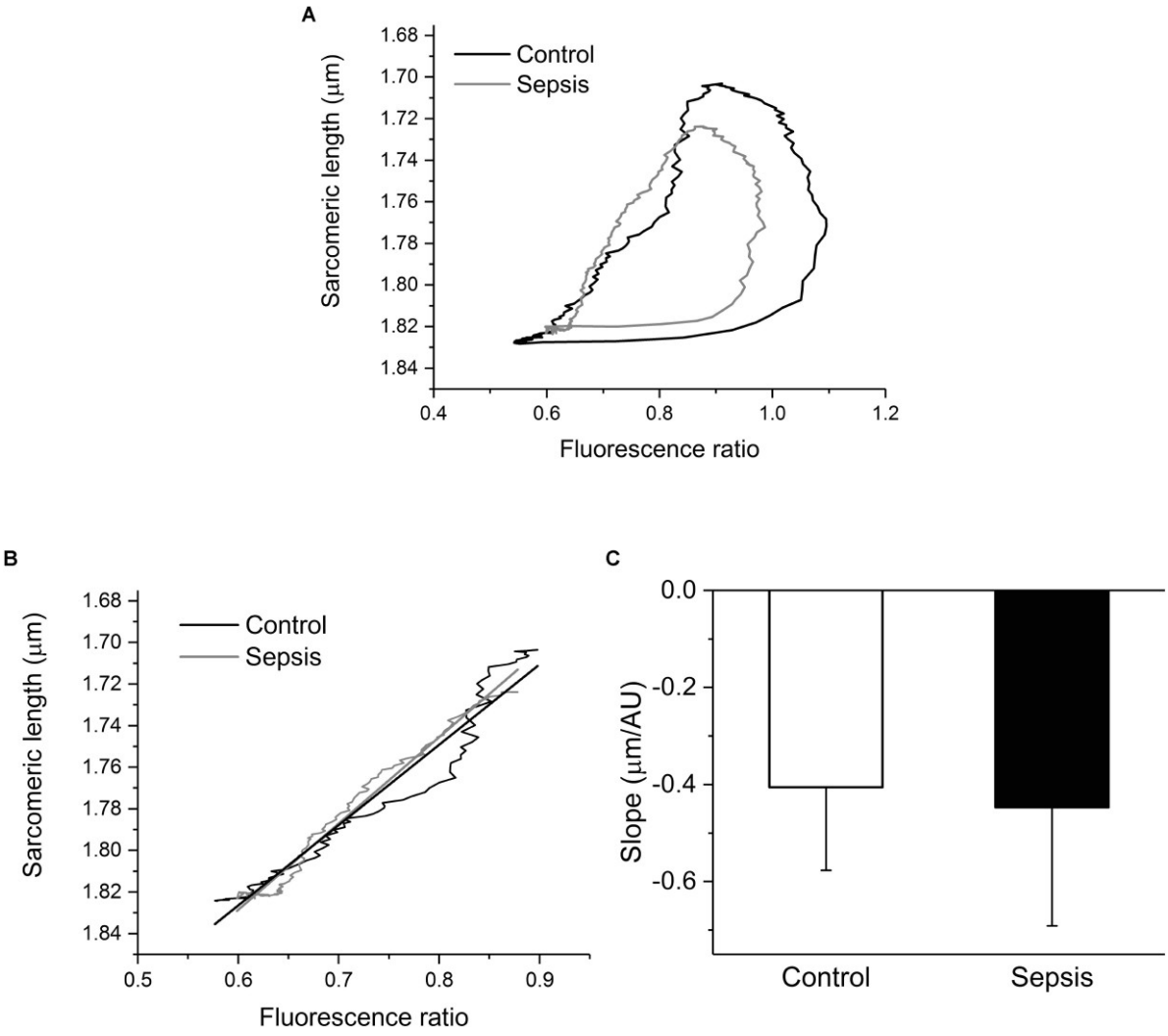

FIGURE 6 | Phase-plane diagrams of sarcomeric length-intracellular Ca^2 relationship. (A) Mean phase-plane trajectories of sarcomeric length- uorescence ratio relationship in control (black line, n = 7) and septic (gray line, n = 8) myocytes. (B) Ampli ed relaxation phase of the mean trajectories with linear ts. Black lines, control; gray lines, sepsis. (C) Slopes of linear ts of relaxation portions of phase-plane trajectories. Empty column, control; lled column, sepsis.

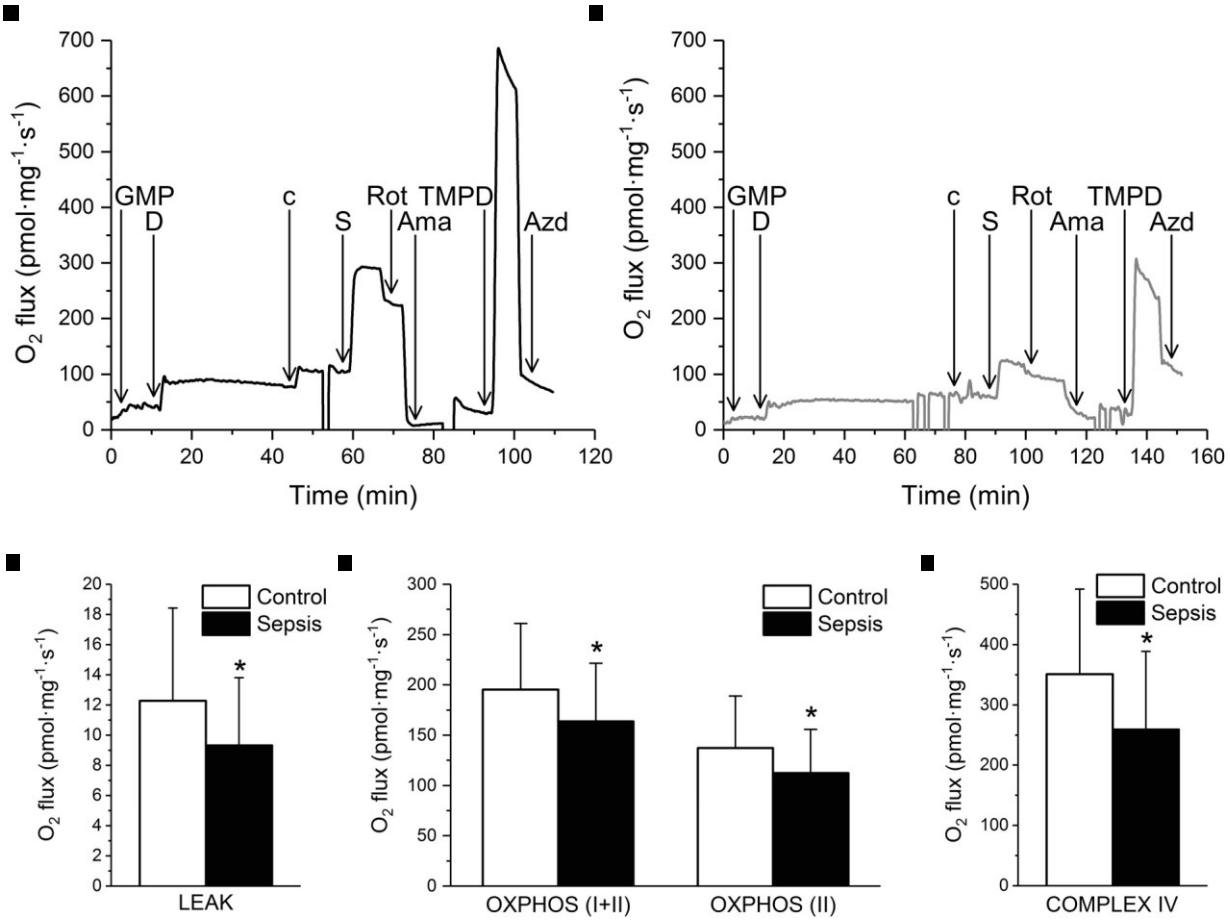

FIGURE 7 | Mitochondrial respiration in sepsis. (A) Representative trace of oxygen consumption during substrate-inhibitor titration protocol in control myocardium. (B) Representative trace of oxygen uxes during substrate-inhibitor titration protocol in septic myocardium. GMP – glutamate, malate, pyruvate, D – ADP, c – cytochrome c, S – succinate, Rot – rotenone, Ama – antimycin A, TMPD – *N,N,N ,N* -tetramethyl-*p*-phenylenediamine dihydrochloride, AZD – sodium azide. (C) Oxygen consumption on LEAK state in control (empty column, $n = 33$ samples/5 pigs) and septic (lled column, $n = 44$ samples/6 pigs) myocardium. (D) Oxygen consumption in OXPHOS (I+II) and OXPHOS (II) states in control (empty columns, $n = 33$ samples/5 pigs) and septic (lled columns, $n = 44$ samples/6 pigs) myocardium. (E) Complex IV oxygen consumption in control (empty column, $n = 33$ samples/5 pigs) and septic (lled column, $n = 44$ samples/6 pigs) myocardium. Signi cantly different from control, $p < 0.05$.

not in uenced signi cantly (**Figure 7D**). Cytochrome c oxidase (Complex IV) activity was also decreased in sepsis (**Figure 7E**).

Citrate synthase activity was not a[glected by sepsis, reaching 64.2 12 and 62.1 11 IU/g in control and septic samples, respectively.

DISCUSSION

In our clinically relevant porcine model of peritonitis-induced sepsis/septic shock, we have managed to induce the typical hyperdynamic circulation pattern, with high heart rate but reduced stroke volume and low systemic vascular resistance. On the cellular level, this was associated with shortened action potential duration, decreased contraction force and calcium transient amplitude, and reduced I_{CaL}. Analysis of phase-plane diagrams of sarcomeric length versus calcium concentration (uorescence ratio) indicated no change in myo lament calcium sensitivity. Mitochondrial respiration was suppressed in septic hearts, predominantly due to an inhibition of Complexes II and IV.

To the best of our knowledge, this is the rst complex analysis of cellular and subcellular mechanisms of septic myocardial depression in a clinically relevant large animal (porcine) experimental model. In earlier studies, the cellular e[glects of sepsis on excitation–contraction coupling in the myocardium were only studied in small animal (rodent) models. Consistent with our porcine data, septic peritonitis rat model cardiac myocytes exhibited a depression of both peak shortening and calcium transients (Ren et al., 2002). Similarly, decreased myocyte shortenings and peak systolic calcium levels, together with slowed-down kinetics of calcium transients, were described in isolated cardiac ventricular cardiomyocytes of rats with sepsis induced by cecal

ligation and puncture (Zhu et al., 2005). In mice with sepsis due to colon ascendens stent peritonitis, cardiac myocytes showed reduced cell shortening, calcium transient amplitude, and sarcoplasmic reticulum calcium content, which was associated with a signi[cant increase in oxidation-dependent calcium and calmodulin-dependent protein kinase II activity (Sepúlveda et al., 2017). In general, the data obtained in rodent models of sepsis/endotoxemia indicate an important role of intracellular calcium homeostasis in septic myocardial depression.

In this study of porcine septic shock, the reduction of cardiac contractile force (documented in multicellular preparations of cardiac trabeculae, as well as in isolated cardiac myocytes) was associated with decreased amplitude of calcium transient, reduced I_{CaL}, and shortened action potential duration. I_{CaL} represents the main entry pathway of calcium into the cardiac myocyte and is crucial for both triggering calcium release from the sarcoplasmic reticulum and replenishing intracellular calcium stores during the plateau phase of the cardiac action potential (Eisner et al., 2017). Reduction of I_{CaL}, together with the shortening of action potential duration, results in a substantial suppression of the calcium in[ux (Stengl et al., 2010). Similar kinetics of the rising phase of calcium transients in control and septic myocytes suggest that triggering of the sarcoplasmic reticulum calcium release was not signi[cantly a[glected by the reduction of I_{CaL}, leaving the diminished I_{CaL} calcium in[ux for replenishing intracellular calcium stores and/or direct stimulation of contractile proteins as the most likely mechanism.

Another possible contributor to septic myocardial depression might be the altered functional properties of myo[lar proteins. In rats with cecal ligation and puncture sepsis, the phosphorylation of both troponin I and of C protein was increased during the early phase but decreased during the late phase of sepsis (Wu et al., 2001). The decreases in the phosphorylation of troponin I and C protein during late sepsis coincided with the declines in the activities of myo[brillar ATPase and the calcium sensitivity of m[t. Similarly, in rabbit non-lethal endotoxemia, a phosphorylation-dependent decrease in myo[brillar calcium sensitivity was documented (Tavernier et al., 2001). In contrast to these [ndings, septic plasma from a canine model of Escherichia coli sepsis failed to decrease isometric tension in the skinned trabecular preparations with chemically disrupted sarcolemmal, sarcoplasmic reticulum, and mitochondrial membranes, which ruled out a direct inhibition of the contractile apparatus by septic plasma (Gu et al., 1998). In line with the canine sepsis data, the phase-plane analysis of sarcomeric length-intracellular Ca^2 relationship revealed no signi[cant change of myo[lament responsiveness in porcine septic shock. The intracellular Ca^2 -cell length trajectory during the relaxation phase of the twitch contraction in single cardiac myocytes de[nes a quasi-equilibrium of cytosolic calcium, myo[lament Ca^2 binding, mechanical force, and cell length (Spurgeon et al., 1992). Since the position and the slope of the relaxation phase trajectories were virtually identical in control and septic myocytes, the calcium responsiveness of myo[laments was probably not a[glected in our experimental setting of porcine sepsis.

The growing consensus that mitochondrial dysfunction contributes to the pathogenesis of sepsis and development of septic cardiomyopathy is mainly based on studies performed in small laboratory animals (Cimolai et al., 2015). Decreased respiratory rates and/or reduced activities of respiratory mitochondrial complexes were reported in septic rabbits (Gellerich et al., 2002), rats (Vanasco et al., 2012), and mice (Piquereau et al., 2013). In majority of studies, inhibition of Complex I was documented, while inhibition of other Complexes was less consistent. Only two studies published so far addressed the myocardial mitochondrial respiratory dysfunction in porcine models of peritonitis-induced sepsis: one of them reported reduced activity of Complex I determined spectrophotometrically at 30 C and expressed per citrate synthase activity (Li et al., 2007); the other documented no di[glerence in myocardial mitochondrial oxygen consumption between control and septic animals treated with antibiotics for at least 48 h (Corrêa et al., 2012). In our current study, suppression of mitochondrial respiration related to Complexes I and II (OXPHOS I II) could be attributed mainly to inhibition of Complex II. Oxygen consumption by arti[cially s[mulated Complex IV was also reduced, while activity of Complex I was not signi[cantly in[uenced. Expression of oxygen c[p[per citrate synthase activity did not a[glect the pattern of changes. Decreased LEAK state with reduced mitochondrial respiration expressed per mg tissue wet weight would suggest reduced mitochondrial content and/or swelling of the tissue due to [uid resuscitation; however, unchanged citrate synthase activity argues against these options. The experimental discrepancies are probably related to species di[glerences, as well as variable experimental protocols (models of sepsis, duration of sepsis, severity of insult). Is there a direct link between abnormal calcium handling and mitochondrial dysfunction? It is well-known that cardiac sarcoplasmic reticulum and mitochondria closely interact, forming a mitochondrial calcium microdomain (Kohlhaas and Maack, 2013). The sarcoplasmic reticulum Ca^2 -ATPase preferentially consumes mitochondrial ATP for active transport of cytosolic calcium back to the sarcoplasmic reticulum (Kaasik et al., 2001). On the other hand, calcium released from the sarcoplasmic reticulum enters the mitochondria, even on a beat-to-beat basis (Andrienko et al., 2009), and regulates mitochondrial enzymes of the tricarboxylic acid cycle, the proteins of the electron transport chain, and the ATP synthase (Williams et al., 2015), thus matching energy supply to demand. Accordingly, in our study, decreased contraction and calcium release were accompanied by decreased mitochondrial respiration, but the question remains as to what the primary event is. In our opinion, the facts that the kinetics of calcium transients decline and the m[t calcium responsiveness were not a[glected by sepsis argue against the primary role of the mitochondria and insu[:icient energy supply, and rather indicate defective calcium transport (reduced I_{CaL} with consequent alterations of sarcoplasmic reticulum calcium release) as the primary event of myocardial depression.

CONCLUSION

Defective calcium handling with reduced calcium current and transients, together with inhibition of mitochondrial respiration, appear to represent the dominant cellular mechanisms of myocardial depression in porcine septic shock. Consequently, these molecular mechanisms may help to identify potential therapeutic targets for preventing and/or reversing sepsis-induced myocardial dysfunction. In this line of thinking, calcium channel openers represent an obvious option. Bay K 8644, a dihydropyridine calcium channel agonist, was shown to enhance I_{CaL} in cardiac myocytes from endotoxemic rats (Abi-Gerges et al., 1999). *In vivo*, BAY K 8644 elevated blood pressure in endotoxin-shocked rats (Ives et al., 1986) as well as in endotoxemic dogs (Preiser et al., 1991). On the other hand, in cardiac myocytes from endotoxemic guinea pigs BAY K 8644 did not reverse the endotoxin-induced reduction in peak I_{CaL}, cell contraction, and systolic intracellular calcium concentration (Zhong et al., 1997). Mitochondrial respiration is another promising target for potential therapeutic interventions. In endotoxemic rat models, mitochondria-targeted antioxidants were demonstrated to reduce in[illegible]ry responses, mitochondrial damage and organ dysfunction including cardiac depression (Supinski et al., 2009; Lowes et al., 2013).

STUDY LIMITATIONS

The modi[illegible]ed SOFA score was determined according to human sepsis criteria (Singer et al., 2016). Despite generally similar physiology and sepsis progression in this porcine model and humans, it is possible that some criteria are not completely compatible and will require further validation in the model. Furthermore, sepsis was induced in young, healthy animals and the translation to elderly intensive care unit (ICU) patients with multiple comorbidities that may a[glect cardiac calcium handling and mitochondrial function themselves (e.g., heart failure) might be di[:icult.

The porcine peritonitis-induced sepsis model shows a clear hyperdynamic phenotype and therefore care should be taken to not generalize the results to the hypodynamic phenotypes. It remains, for future studies with more appropriate models, to determine whether or not and to what extent the above described mechanisms contribute to hypodynamic sepsis.

The upstream mechanisms that induce cardiac cellular alterations were not addressed in this study. Extensive research of possible candidates and signaling pathways (e.g., cytokines, oxidative and nitrosative stress, circulating histones) is clearly warranted.

AUTHOR CONTRIBUTIONS

JH, LN, and JB conducted the *in vivo* experiments, DJ, JS, LN, MS, and MA participated in cellular and tissue cardiac experiments; MiM and JK conducted the mitochondrial experiments, and MaM and MS conceived of and designed the study and drafted the manuscript. All authors participated in interpretation of the studies, analysis of the data, review of the manuscript, and read and approved the [illegible]nal manuscript.

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REVIEW

Variability of Mitochondrial Respiration in Relation to Sepsis-Induced Multiple Organ Dysfunction

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Summary

Ample experimental evidence suggests that sepsis could interfere with any mitochondrial function; however, the true role of mitochondrial dysfunction in the pathogenesis of sepsis-induced multiple organ dysfunction is still a matter of controversy. This review is primarily focused on mitochondrial oxygen consumption in various animal models of sepsis in relation to human disease and potential sources of variability in experimental results documenting decrease, increase or no change in mitochondrial respiration in various organs and species. To date, at least three possible explanations of sepsis-associated dysfunction of the mitochondrial respiratory system and consequently impaired energy production have been suggested: 1. Mitochondrial dysfunction is secondary to tissue hypoxia. 2. Mitochondria are challenged by various toxins or mediators of inflamrnation that impair oxygen utilization (cytopathic hypoxia). 3. Compromised mitochondrial respiration could be an active measure of survival strategy resembling stunning or hibernation. To reveal the true role of mitochondria in sepsis, sources of variability of experimental results based on animal species, models of sepsis, organs studied, or analyt:ical approaches should be identified and minimized by the use of appropriate experimental models resembling human sepsis, wider use of larger animal species in preclinical studies, more detailed mapping of interspecies differences and organ-specific features of oxygen utilization in addition to use of complex and standardized protocols evaluating mitochondrial respiration.

Keywords

Sepsis • Mitochondria • Oxygen consumption • Multiple organ dysfunction • Animal models

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Introduction

Sepsis, lit'e-threatening organ dysfunction caused by a dysregulated host responsc to infection, represents one ofthe most serious public health issues ofthe modem time. In the United States it affects more than 750,000 patients per year (Angus *et al.* 2001); its incidence is similar in Europe and probably even higher in lo w-income countries (Fleischmann *et al.* 2016). Mortality rate ranges between 30-70 % in re lation to severity of the disease, paiticularly to the number of dysfonctional organs (da Silva *et al.* 2008).

Definition of sepsis

ln tbe coursc ot' past few decades, definition ot' sepsis has substantially changed. Originally, it was based mainly on the evidence of bactere1nia or toxemia. In 1991 , sepsis-1 defiuition was establishedas an infcction or suspected infection lea di ng to the onset of systemic inflamrnatory rcsponse syndrome (SIRS). SIRS diagnostic criteria (Table 1) were based on tbe precisely defined changes in body temperature, heart rate, respiratory functions, and white blood cell count (Bone *et al.* 1992). In addition, severity of the disease was classified in three categorics - sepsis (present/suspect

infection + at least 2 SIRScriteria,)severe sepsis (disease 2017). This sepsis-3 definition is based on bettcr complicated by organ dysfunction) and scptic shock understanding of the pathophysiology of the disease and ("sepsis-induced hypotension persisting despite adequate emphasizes maladaptive host response to infection that fluid resuscitation"). Criteria of SIRS were furthcr results in organ dysfunction (Singer *et al.* 2016). Presence expanded in sepsis-2 defuútion in 2001 (Levy *et al.* of SIRS is not further rcgarded as a crucial marker of 2003). More substantial modification of definition of sepsis as it could be associated with a desired response of sepsis was approved in 2016 at a consensus conference of the body to the int'ection challenge. Consequcntly, the Society of Critical Care Medicinc and the European ca.tegory ofsevere sepsis became needlcss.
Society of Intensive Care Medicine (Marik and Taeb

Table 1. Definitions of sepsis.

SEPSIS-1 (1992) Suspected/documented infection + SIRScriteria	**SEPSIS-2** (2003) Suspected/documented infection + extended STRS criteria	**SEPSIS-3** (2016) Suspected/doc umented infection + SOFA score
SIRS criteria • Temperature • Heart rate • WBCC/Bands • Respiratory rate/$PaC0_2$ **Organ dysfunction** SBP, MAP, coagulation, bilirnbin concentration, urine output, crea tinine concentration, oxygen saturation	**Extended SIRS criteria** • **General parameters** Tempe rature, heart rate, respiratory rate, hyperglycemia without DM, altered mental status, edcma • **In0ammatory parameters** WBCC/Bands, CRP, procalcitonin • **Hemodynamic parameters** MAP, SBP, 1nixed venous oxygen saturation, cardiac index • **Organ dysfunction** $Pa0_2$, urine output, creatinine concentration, coagtdation, plate let count, liver fi.mction (bilirubin concentration), gastrointestinal motility • **Tissue perfusion** Lactate concentration, capillary refill	**SOFA** • $Pa0_2/Fi0_2$ ratio • Glasgow coma scale score • MAP • Vasoprcssors • Serum crea.tinine • Bilirubin • Platelet count
Staging **Sepsis** = infection + 2 SIRS **Severe sepsis** = sepsis + organ dysfunction, hypoperfitsion, or hypotension **Septic shock** = sepsis + hypotcnsion despite adequate fluid rcsuscitation + hypoperfusion or organ dysfm1ction	**Staging** **Sepsis** = infection + 1 extcnded SIRS **Severe sepsis** sepsis + organ dysftmction **Septic shock** = sepsis + refractory hypotension tmexplained by other causes	**Staging** **Sepsis** life-threatening organ dysfi.mction caused by a dysregulated host response to infection **Septic shock** = a subset of sepsis in which profound circulatory, cellula.r and metabolic abnormalitics are associated with a greater risk of mortality than wi th sepsis alone

Variables evaluated in diagnosis of sepsis. SIRS - systemic inflammatory response syndrome, WBCC - white blood cell count, PaC02 - arterial partial pressure of C02, SBP - systolic blood pressure, MAP - mean arterial pressure, DM - diabetes mellitus, CRP - C-reactive protein, PaOi - arterial partial pressure of *Oi,* SOFA - sepsis-related (sequential) organ failure assessment score, Fi02 - fraction of inspired 02.

Pathophysiology of sepsis

Scpsis is characterized by homeostatic dysbalance that could progress in mLLltiplc organ dysfi.mction (MOD), septic shock and dcath. Pathogen-associatcd molecular pattcm (PAMPs) stimulatc plasma complcment and coagulation systems and bind to pattem recogrution receptors on the cel! membranes and/or intraccllular organelles. The simultaneousattack on the multilevel body's defense mechanisms provokes both passive and active release of dangcr-associated molecular pattems (DAMPs) from dying cells or cells chaUenged by PAMPs, like heat shock proteins, genomic and mitochondrial DNA, or ATP (Sharma and Naidu 2016) and others. These molecules fi.uther invade the host's tissues causing increased expression and release of a number of inflamrnatory mediators and biomarkers including those that promote inllarnmatory response, those that fight against infection, membrane rcceptors and their downstream effectors, molecllles released from damaged cells, chemicals associated with activation of the coagulation and complement systems, vasoactive substances, acute phase proteins, biomarkers of various organs dysftmction, and others (Angus and van der Poll 2013). To date, about 180 biomarkers of sepsis have been identified (Pierrakos and Vincent 2010). Dysregulated immune reaction along wit:h hormonal dysbalance, distLrrbed activity of the autonomie nervous system and dysftmction of epithelial and endothelia! cells lead to the loss of barriers tightness, changes in intermediary metabolism, and subsequently to organ dysfunction. MOD is frequently manifested by respiratory clistress, myocardial depression, systemic vasodilatation, acute kidney ittjmy, impaired liver function, distLrrbed gastrointestinal motility, and coagulopathy (Reinhart *et al.* 2012, Fig. 1).

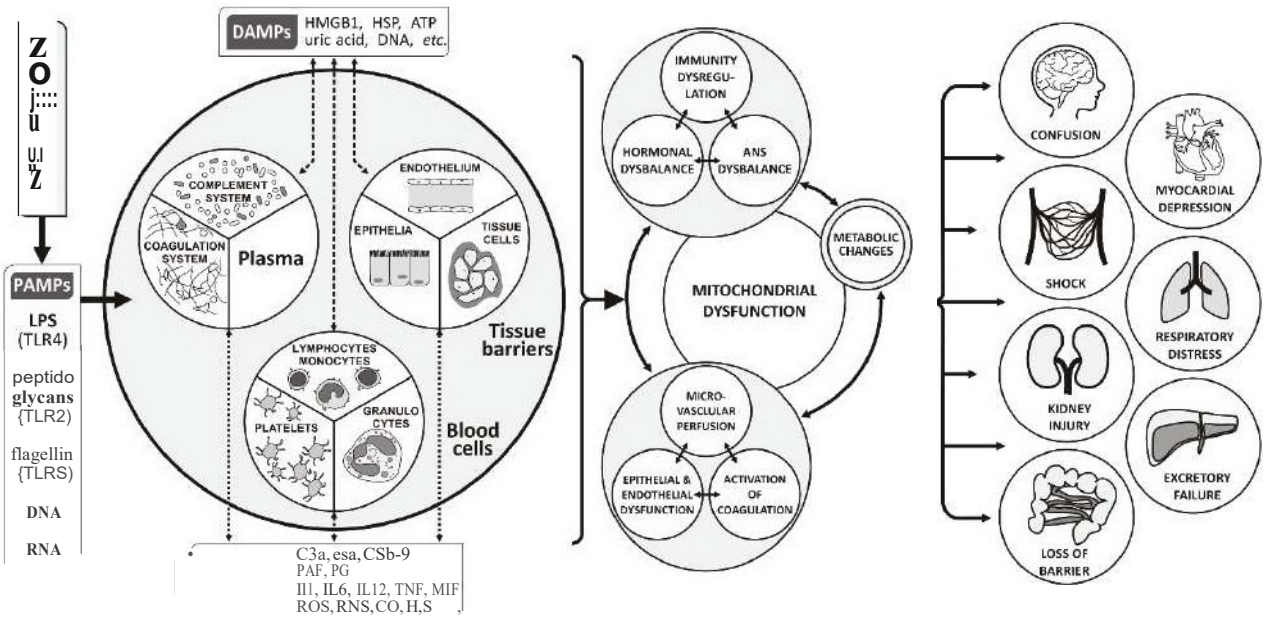

Fig. 1. Pathophysiology of sepsis. Pathogen-associated molecular pattems (PAMPs) bind to pattern recognition receptors in the cel! membranes and intraeellular organelles. Subsequent release of danger-associated molecular patterns (DAMPs) invading the host's tissues causes increased expression and release of inflammatory mediators and biomarkers. Dysregulated host response to the septic insult includes disturbed immune, endocrine, and autonomie nervous systems regulation, loss of barriers tightness, changes in intermediary metabolism, and subsequently multiple organ dysfunction. PAMPs - pathogen-associated molecular patterns, LPS - lipopolysaecharide, TLR - Toll-like receptor, DNA - deoxyribonucleic acid, RNA - ribonucleic acid, DAMPs - danger -associated moleeular patterns, HMGBI - high mobility group box 1, HSP - heat shock protein, ATP - adenosine triphosphate, PAF - platelet -activating faetor, PG - prostaglandins, IL - interleukin, TNF - tumor necrosis factor, MIF - macrophage migration inhibitory factor, ROS - reactive oxygen species, RNS - reaetive nitrogen species, CO - carbon monoxide, H2S - hydrogen sulfide, ANS - autonomie nervous system.

Mitochoodria in health

Mitochondria are scmi-autonomous cel! organelles that are primarily designed to produce bi.olo gicallyavailable energy in the fonu of adenosine triphosphate (ATP). The inner mitochondrial membrane houses a sophisticatcd electron-transport system (ETS) designed to transfer electrons from appropriate substrates sequentially across multiheteromeric respiratory complexes (I to IV) and two mobile elements (coenzyme Q and cytochrome c) to their final acceptor,

molecular oxygen (Fig. 2A). Gradually released energy is used to pump protons from the mitochondrial matrix into the intennembrane space thus gcnerating a proton gradient driving ATP synthesis by complex V, ATP-synthase (Fig. 2). The substratcs fueling directly or indirectly oxidative phosph01ylation are mainly produced in the mitochondrial rnatrix in crncial metabolic processes, i.e. tricarboxylic acid (TCA) cycle and -oxidation offatty acids (Gnaiger 2014). Electrons for ETS are also provided by pyruvate dehydrogenase complex, glutamate dehych·o-genase, sulfite oxidase (Velayutham *et al.* 2016), sulfide ubiquinone oxidoreductasc, clihydroorotate dehydrogenase (Lemieux *et al.* 2017) or mitochondrial glycerol phosphate dehych·ogenase (Mráček*et al.* 2013, Fig. 28).

Bcsidcs their key role in energy production, mitochondria are also site of production of steroid honn ones, heme, iron-sulfur clusters, and endogenous gases including reactive oxygen and nitrogen species that are required for cellular signaling. Mitochondria are also conside red important regulators oť intrac e llular calcium concentration; they generate heat thus contributing to temperature regulation and basa! metabolic rate, initiate apoptotic cell death and may also have critical and multiple functions in the initiation oť cell cliff e re n t iation, ce ll-type detemúnation, cell movement, and pattem formation (Maeda and Chida 2013).

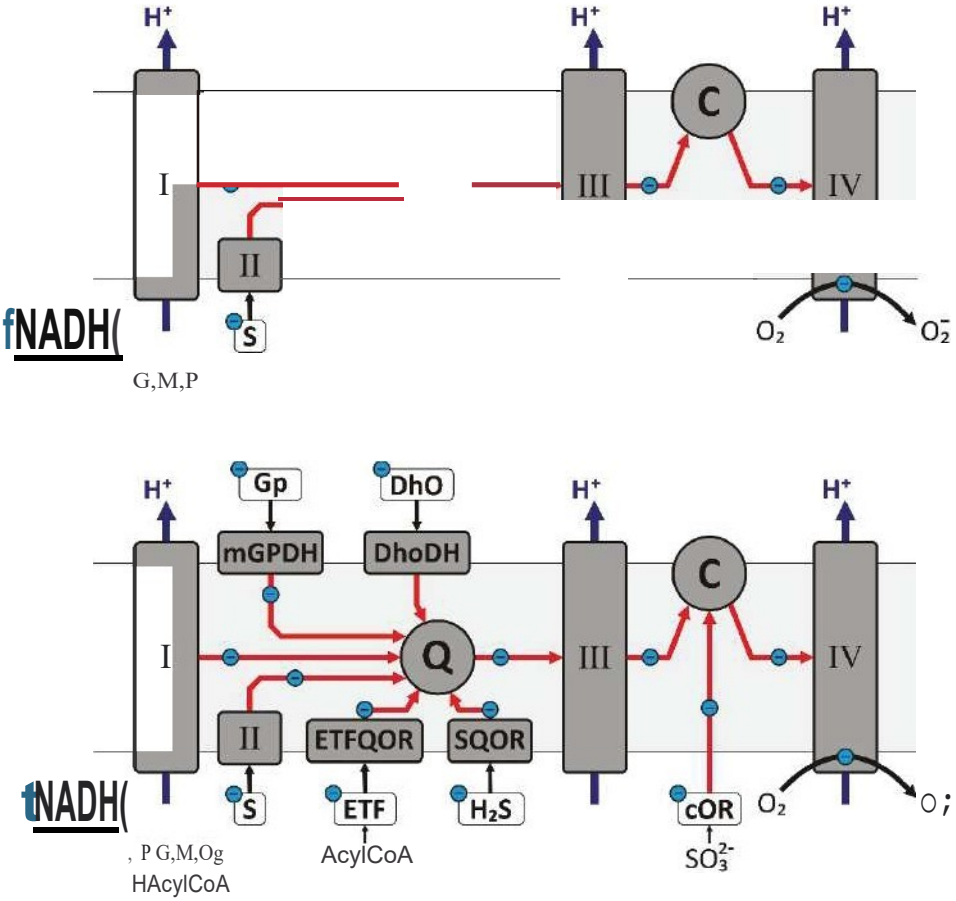

Fig. 2. Mitochondrial electron transport. Electron-transport system (ETS) serves to transfer electrons from substrates sequentially across four respiratory complexes (I to IV), two mobile carriers (coenzyme Q and cytochrome c) to final electron acceptor, molecular oxygen. **(A)** Simplified version of ETS reflecting widely used experimental protocols evaluating mitochondrial oxygen consumption. **(B)** More complex view on ETS including additional electron donors. Electron transport is coupled with proton pumping from the mitochondrial matrix into the intermembrane space thus generating a proton gradient driving ATP synthesis by complex V, ATP-synthase. Electrons fueling ETS come from substrates produced in tricarboxylic acid cycle and oxidation of fatty acids, from pyruvate dehydrogenase complex, glutamate dehydrogenase, mitochondrial glycerol phosphate dehydrogenase, sulfrt:e oxidase, dihydro-orotate dehydrogenase, sulfide-ubiquinone oxidoreductase, and choline dehydrogenase (not shown). II, III, IV- complexes of electron-transport system, e - electron, *W* - proton, Q - coenzyme Q, c - cytochrome c, *Oi* - oxygen, NADH - nicotinamide adenine dinucleotide, G - glutamate, M - malate, P - pyruvate, 0g - oxoglutarate, HAcylCoa - hydroxyacyl-coenzyme A, S - succinate, Gp - glycerol phosphate, DhO - hydroxyorotate, H2S - hydrogen sulfide, mGPDH - mitochondrial glycerol phosphate dehydrogenase, AcylC.OA - acyl coenzyme A, ETF - electron transferring flavoprotein, SQOR - sulfide:quinone oxidoreductase, ETFQOR - electron-transfer flavoprotein:ubiquinone oxidoreductase, cOR - cytochrome c oxidoreductase, so/·- sulfite anion.

Mitochoodria in sepsis

Multiple experimental data on animals as well as humans suggest that exaggerated inflammatory response could intcrfcre with any mitochondrial function; i.e. appropriate production of reactive oxygen and nitrogen species (recently rev iewed by Duvigneau and Kozlov 2017), intracellular calcium homeostasis (Pinto *et al.* 2017), mitochondrial biogenesis and tumover (Inata *et al.* 2018), or regulation of apoptosis (Chen *et al.* 2017). This review is primarily focused on 1nitochondrial oxygen consumption in various animal models of sepsis in relation to human disease. Despite intense research in this field, the precise role of mitochondria in the chain of events leading to MOD is still a matter of controversy. So far reported experimentaJ data on changes m mitochondrial respiration are far from being uni fom1 and seem to depcnd on a number of factors including the organ studi ed, selected model of sepsis, animal species, severity and phase of the disease, and the experimental set-up (Singer 2014). Interestingly , despite frequently suggested key role of mitochondrial bio energ etics in the development and progression ofMOD, the most frequent finding in experimental studies dealing with mitochondrial respiration or respiratory enzymes activities in various rodent models of sepsis was unchanged oxygen consumption by the heart, liver and skeletal muscle mitochondria (Jeger *et al.* 2013). However , it should be noted that similar number of s111dies reporte d sepsis-associated decrease or even increase in mitochondrial oxygen consumption in above mentioncd organs (Jcger *et al.* 2013) and that unchanged respiration does not necessarily mean that the energy production is appropriately adjusted to the tissue needs (Dyson and Singer 2011). In general, there are at least three possible explanations of scpsis-associated dysfh n c tion of the mitochondrial respira to ry system and consequently impaired energy production (Fig. 3):

I . Increased oxygen demand associated with immune system activation, elevated body temperature, and increased metabolic rate together with impaired diffusion processes in microcirculation lea d to tissue hypoxia that is reflected by decreascd oxygen consumptiou in the mitochondria and impaired ATP generation (Kozlov *et al.* 2017). In such view, mitoc hondria would be victims of pathological processes initiated elsewhere and would only follow inadequate oxygen delive1y with insufficient energy production. As reviewed by Chioléro *et al.* (1997), sepsis is frequently associated with increascd metabolic rate, protein and fat catabolism, negative nitrogen balance, hyperglycemia, and insulin resistance. However, these metabolic changes are not conelated with the body temperature and severity of the disease, nor they seem to be in causa! relationship to hyperdynamic pattern of circu lat ion , typical for SIRS in hmnans (Kreymam1 *et al.* 1993). Although alterations in microcirculatory blood flow have been frequently identified in sepsis (De Backer *et al.* 2002), true tissue hypoxja manifested by decreased tissue p0 $_2$ was regularl y rep ort ed mainly in hypodynamic stages ot' sepsis or short-term rodent models of endotoxemia (Dyson *et al.* 2011), where decline in tissue perfusion resulting in decreased tissue oxygen tension could be regarded as a predictable consequence (Matejovic *et al.* 2011). In addition, tissue p0 $_2$ does not reflect onJy oxygen delivery to tissues, but rather a balance between oxygen supply and its cellular co nsumption. Hypoxie theory of mitochondrial dysfunction in sepsis was challenged by studies demonstrating no effect of improved tissue perfusion on the recove1y from sepsis (Gattinoni *et al.* 1995, Hayes *et al.* 1997). Taken together, despite an ample evidence of individua! factors contributing to the aforementioned chain of events leading to an energy cris is during sepsis, thcir poor co1Telation argues against the simple passive role of mitochondria in the progression ofthe disease.

2. Mitochondria could be directly challenged by DAMPs and mediators of inflamma tion that cause decrease in oxygen consumption in conditions of pre serv ed tissue blood flow and oxygen tension. The putative inability of mitochondria to consume oxygen as a mechanism coutributing to organ dysfunction in sepsis was suggested by Fink and termed cytopathic hypoxia (Fink 1997).

As mcntioned above, tmchanged or even incr ease d tissue oxygen tension was repeatedly reported in the skcletal muscles of septic patients (Boekstegers *et al.* 1991, Boekstegers *et al.* 1994) as well as decreased oxygen consumption and ATP generation by ske le tal muscle mitochondria (Brealy *et al.* 2002, Fredrikssou *et al.* 2006), findings that would fit the theory of cytopathic hypoxia. However, data obtained from critical tissues in animal resuscitated models are less convincing. For example, conflicting results we re obtained from thc liver, intestinal mucosa or kidneys where both decreased and unchanged tissue p0 $_2$ were documented (Lund *et al.* 1995, Dyson *et al.* 2011). In addition, results of studies on mitochondrial respiration in these organs display

strong variation thus not providing compelling evidence of impaired oxygen utilization preceding ove1t organ dysfimction (Patil *et al.* 2014, Porta *et al.* 2006). Nevertheless, numerous data suggest that mitochondrial oxygen processing could be significantly affected in response to vario11S mediators of inflammation. Among them, reactive oxygen and nitrogen species, carbon monoxide and hydrogen sulfide ha:ve drawn more attention due to their site of origin and qtůte consistently reported dual effect on mitochondrial electron transport (Duvigneau and Kozlov 2017, Kozlov *et al.* 2017 , Módis *et al.* 2014).

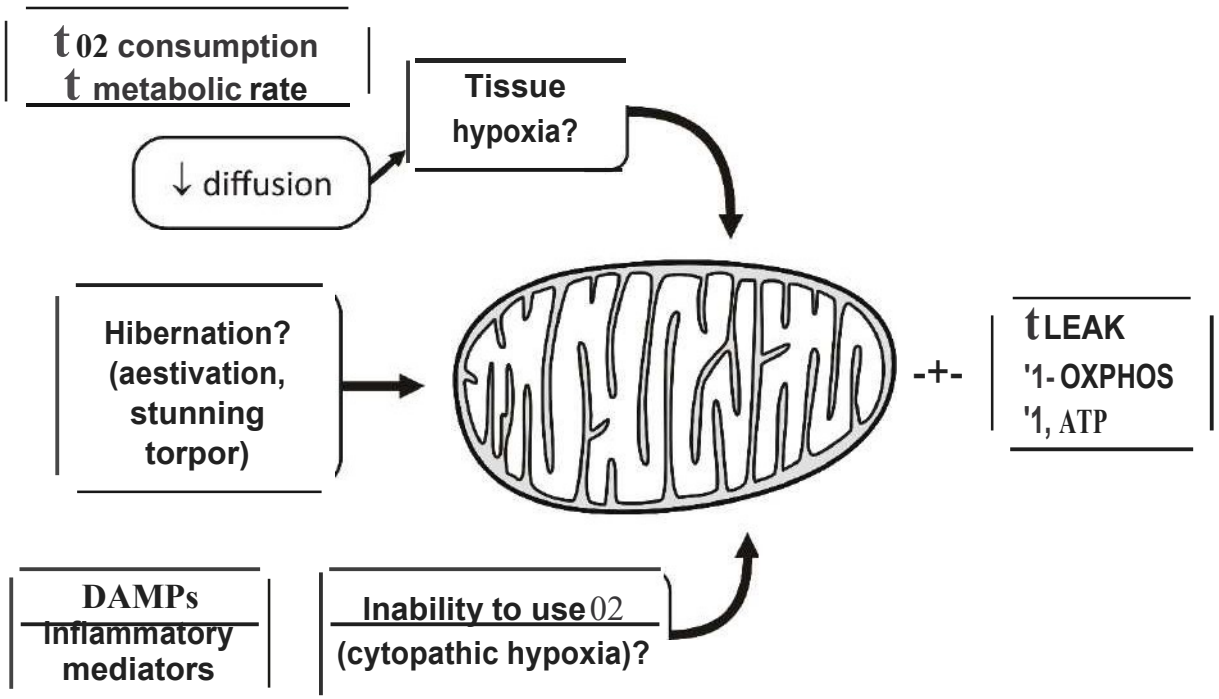

Fig. 3. The role of mitochondria in sepsis - 3 possible explanations of sepsis-associated dysfunction of the mitochondrial respiratory system and energy production. 1. Increased oxygen demand together with impaired diffusion processes in microcirculation leads to tissue hypoxia manifested by decreased oxygen consumption and impaired ATP generation. 2. Mitochondria could be direc.tly challenged by DAMPs and mediators of inflammation that cause decrease in oxygen consumption in conditions of preserved tissue blood flow and oxygen tension. 3. Reduced oxygen consumption could be related to the active role of mitochondria in orchestration of survival strategy resembling stunning or hibemation. These processes could lead to an increase in the mitoc.hondrial respiratory state LEAK, decrease in oxidative phosphorylation (OXPHOS) and limited ATP production. DAMPs - danger -associated molecular patterns, 02 - oxygen, ATP - adenosine triphosphate).

3. Reduced oxygen consumption could be related to the active role of mitochondria in orchestration of survival strategy resembling stunning or hibemation, characterized by regional contractile dysfunction of myocardium due to compromised oxygen supply (Singer 2017). Both phenomena werc originally dcscribed in the hearts subjected to ischemia. The tenn stmmed myocardium was initially used to characterize an abnomial contractile fonction of the left ventricle persisting for hours or days after coronary occlusion lasting not more than 15 min and not being accompanied with cell death (Bram1wald and Kloner 1982 , Hcyndrickx *et al.* 1975). Hibemation was related to chronically compromised coronary perfusion associated with impaired left ventricular fonction that could be complctely restored if blood flow was rcturned to norma! (Braunwald and Rutherford 1986 , Rahimtoola 1993). Ischemia-induced hibemation is regarded as a regulatory measure that maintains myocardial integrity and viability (Heusch *et al.* 2005). At the cellular level, both mechanisms overlap being accompanied with metabolic adaptation characterized by shifting encrgy production away from oxidative phosphorylation and oxygen utilization. Many features of this metabolic adjustment have been identified also in human and mouse carctiomyocytes challenged by sepsis or endotoxemia including dowmcgulation of enzymcs in TCA cycle, suppression of catabolism of energy substrates including fatty acids, glucos e and ketone bodies, accumulation of glycogen and triacylglycerols in the heart (Matkowich *et al.* 2017 , Umbarawan *et al.* 2017), elevated pyruvate dehydrogenase kinase activity leading to suppression of

pyruvate dehydrogenase (Standage *et al.* 2017) or decreased mitochondrial biogencsis (Lancel *et al.* 2009). *In* contrast, no changes in the levels of TCA intermediates and rather increased than decreased mitochondrial biogencsis were detected in thc hearts of septic rats (Hotchkiss *et al.* 1991, Vanasco *et al.* 2014). More importantly, studies dealing with recove1y from sepsis suggested that better energy-producing fatty acid catabolism was associated váth survival of the fittest in sepsis (Langlcy *et al.* 2013) and that PPARa expression (peroxisome proliferator-activated receptor alpha - marker of mitochondrial biogenesis) and increased fatty acid oxidation were associated with the byperdynamic cardiac response early in the course of sepsis and decreased morbidity and mortality (Standage *et al.* 2017). Some clinical studies on sepsis-induced myocardial depression indicated that left ventricular systolic (but not diastolic) dysftmction could be associated with improved outcomc of sepsis (Jardin *et al.* 1999 , Landesberg *et al.* 2012, Parker *et al.* 1984), however, recently published systematic review and meta-analysis ofthe data on newly diagnosed left ventricular systolic dysftmction in criticaUy ill patients admitted to the intensive care tmit with severe sepsis or septic shock concluded that the presence of new left ventricular systolic dysftmction is neither a sensitive nor a specific predictor of mortality (SevillaBeITios *etal.* 2014).

ln conclusion, altbough numerous data indicate substantial role of mitochondria in scpsis-induced MOD, none of the proposed hypotbeses seems to be supported by convincing experimental and clinical evidence.

Poteotial sources of variability

ln the literature, dysfunctional mitochondrial respiration is usually reported as universa! feature of sepsis-induced organ failure and conclusions are made across the animal species, models of sepsis , analyzed organs or cells, and methods used for analysis of mitochondrial oxygen consumption. For example, the classical sn1dy by Brealey *et al.* (2002) performed on skclctal muscle biopsies taken from 28 septic patients and 9 controls recruited from orthopedic department showed au association between nitric oxide overproduction, antioxidant depletion, mitochondrial dysfunction, and decreased ATP concentrations that related to severity of the disease and eventual outcome. However, results and conclusions from this important work (646 citations in WoS) were taken as applicable on mitochondrial function of organs cmcial in the development of MOD, like kidneys or heart (Dennis and Wittig, 2017, Gomez *et al.* 2014, Martin *et al.* 2017). Experimental sn1dies dealing with mitochondrial respiration in these organs are rclatively numerous for heait, but rai·e for kidneys (Jeger *et al.* 2013) and report conflicting results. The putative sources of considerable vaiiability of sepsis-related mitochondrial oxygcn consumption are discussed below.

1 ln humans, *sepsis per se* is an extremely variable entity due to heterogeneous genetic backgrotmd, frequent co-morbidities (diabetes mellitus, chronic kidney injmy, heart failure, tumors etc.), physical fitness, age, source and degree of the triggering insult and its type (Christaki and Giamarcllos-Bourboulis 2014, Giraldo *et al.* 2009, Villar *et al.* 2004). In addition, therapeutic inte1ventions could have dual effect on metabolic tumover and mitochondrial respiration (Chioléro *et al.* 1997).

2 *Species dijferences.* Expcrimental animals used in the research on sepsis include a number of mammalian species from smalt rodent<; to pigs and primates (Fink 2014). At the end of Apríl 2018, a simple query "given species and sepsis" in PubMed provided 13,000 /9,000/2,800 /2,400 /2,000n oo/300 hits for mouse/ rat/dog/pig/rabbit/cat/baboon (reviews not excluded) witli the highest counts for mouse (approx. 500-800 hits per year over the past 10 years). Tbe use of laboratory rodents (namely mouse and rat) has been challcnged by a number of studies pomtmg out substantial morphological and functional differences that could be related to discrepancies in the progression and outcome of scpsis between these species and humans. Besides well-known differences in tbe life-span, body weight, anatomy and functions of the organ systems (cardiovascular, urina1y, gastrointestinal- Beuchat 1990, Kararli 1995, Milani-Nejad and Janssen 2014) , species-dependent variations in the genetic backgrotmd (Seok *et al.* 2013), immune system (Mestas and Hughes 2004), intermediary and drug met.abolism (Fujiwara *et al.* 2018, MacDonald *et al.* 2011, Mathew *et al.* 2017), production of reactive oxygen species (Barja 2007) , susceptibility to infection (Cross *et al.* 1993), or ability to enter tbc metabolic torpor response (Schubert *et al.* 2010) might be directly related to poor outcome ot' clinical trials documenting no benefit or even deleterious impact of agcnts that exerted promising effects in animal experiments (Deitch 2005, Fink 2014).

3 *Mode/s of septic insult.* Sepsis is an enormously complex condition that is difficult to be

reproduced in experimcn tal setting (Marshall *et al.* 2005). Sclected aspects of processes associated with the impact of the disease on individua! cell types can be studied *in vítro* on cultured tissue cells or freshly isolated blood cells challenged by septic plasma (Mariano *et al.* 2008, Sjovall *et al.* 20 IO). Blood cells isolated from septic patients (leukocytes and platelets) also represent the onJy easily available biological material that can be used to measure mitochondrial oxygen consumption in humans (Belikova *et al.* 2007, Puskarich *et al.* 2016). However, applicability of tbc results of these studies on mitochondrial functions of solid organs is timjted as they are not in direct contact with blood. Leukocytes and platelets are also those cells that are considered major producers of many inflammatory mediators. Morc complex imitation of septic conditions can be achieved using *in vivo* animal models. Of note, it is extremely difficult to find more than two research groups using exactly the samc animal model of sepsis. Besides aforementioned animal species used in sepsis research, the method of disease induction, performance and timing of resuscitation and sampling, age and sex of experimental animals, their genetic background and absence of complicating diseases represeut factors contributing to the extreme variability of the research results in the field of sepsis-induced mitochondrial dys:fonction (Singer 2007). Over the past two decades, various models of sepsis on experiment.al animals have been extensively reviewed (Deitch 1998, Freise *et al.* 200I, van der Poli 2012, Zanotti-Cavazzoni and Goldfarb 2009) with emphasis on their relevance to human disease (Dyson and Singer 2009, Esmon 2004, Poli-dc-Figueiredo *et al.* 2008, Rittirsch *et al.* 2007), translational potential (Dejager *et al.* 2011, Fink 2014, Pitts aud Simpson 2010), particular process to be studied (Doi *et al.* 2009, Fink 2008, Siempos *et al.* 2014) or arumal species used (Lewis *et al.* 2016, Redl and Bahrami 2005, Stortz *et al.* 2017). The list of some biological models used in sepsis research is shown in Table 2.

4 *Mitochondrial diversity.* Mitochondrial DNA diversity is a well-known phenomenon used to make various inferences about the origins of modem humans (Cann *et al.* 1987, Stoneking and Soodyall 1996), phylogeny (Htust and Jiggins 2005) or in forensic analysis (Melton and Nelson 200l). Tissue diversity of mitoc hondrial morphology, quantity and composition has been reported in mouse tissues (Mootha *et al.* 2003, Pagliarini *et al.* 2008). An excellent study by Benard *et al.* (2006) determined the composition and functional features of the respirato1y chain in nmscle, heart, liver, kidney, and brajn of male Wistar rats and concluded that tissues could be categorized at least into three groups: muscle and heait, brain, and liver and kidney. Tissue-dependent values of mitochondrial oxygen consumption were also repo1ted in porcine skeletal muscle, liver, and kidney (Porta *et al.* 2006) and rat hea1t, liver, brain and kidney (Pecinová *et al.* 2011). It is thus clear that conclusions about sepsis-driven changes in mitochondrial oxygen consumption made across different organs could be misleading.

5 *Analytical methods.* Although mitochondrial oxygen consumptiou would seem to be a robust and stable parameter if given experiment.al conditions are maintained, reproducibility and comparabiljty of mitochondrial analyses dcpend on a numbcr of factors that arise from the preparation of organelles and the methodological approach used. Oxygen consumption could be studied on isolated mitochondria, tissue homogenates, isolated cells or permeabilized tissue samples (Kuznetsov *et al.* 2008). Tso lation of mitochondria by differential centrifugation could lead to loss of organelles that were damaged by pathological proccsses induced by sepsis and subsequent determination of oxygen consumption then could give misleading results (Piper *et al.* 1985). Measurement of mitochondrial respiration on the whole cells is swtable for blood cells, isolated hepatocytes or cultured tissue cells, quality of which can be dependent on isolation procedure (Frezza *et al.* 2007). In particular, platelets and white blood cells can be stimulated by mechanical marupulation and contact with media and laboratory plastics (Kramer *et al.* 2014). Mecharucal and chemical penneabilization of the tissue is well standardized for skeletal and cardiac muscles, repo1ted for the liver tissue, but not yet available for other organs. Tissue homogenates are widely used in the evaluation of mitochondrial respiration; however, the homogenization itself is ilifficult to be standarilized and can damage intracellular organelles (Cantó and Garcia-Roves 2015).

Activity of individua! respirato1y complexes can be detennined using methods based on analysis of oxygen consumption (using classical oxygraphy, high resolution respirometry, or fluorescent probes) in frcshly dissected tissue or isolated mjtochondria or by classical spectrophotometric methods determirung their enzymatic activities (Peny *et al.* 2013). *ln vivo,* mitochondrial respiration is substantially dependent not only on tbc ability of ETS to transport clectrons and to couple it with

phosph01ylati.ng process, but also on the availabili.ty of substrates and presence of regulatory factors (Ozkok *et al.* 2016, Schopf *et al.* 2016, Tantama *et al.* 2013). Such a sit uation is diffictLlt to be reprodu ced in *ex vivo* conditions. In classical oxygraphy or more sophisticated high resolution resp irometry, saturating concentrations of substrates are provided (Pesta and Gnaiger 2012) , whereas in the living cells in tissues challenged by SIRS, availability of natmal substrates could be challenged (Bar-Or *et al.* 2018, Waltz *et al.* 2016). In addition, various oxygraphic protocols only pa1tly reproduce the situation *in vivo,* where the substrates providing electrons to ETS are more nurnerous. ln the most frequent protocol used to evaluate activity of complex I , only glutamate and malate are used as substrates providing NADH, although the real situation is much more variable (Fig. 28). For example, combinations of substrates malate + glutamate could give different results than malate + pyrnvate (Gnaiger 2009 , Lemieux *et al.* 2017). The total oxygen consumption by mitochondria is not dependent only on convergent flow of electrons from complexes I and lJ on coenzyme Q, but could be also affected by functional in tegrity of elect ron transporting flavoprotein and other electron donors that are not regularly included in analyses of mitochondrial respiration , like mGPDH (Ramos-Filho *et al.* 2015 , Rauchová *et al.* 2014). In addition , the parameter determined by oxygraphic techniques after addition of substrat es and ADP might not be trne oxygen consumption by in situ mitochondria, but capacity ofETS to consume oxygen under coupled conditions (i .e . OXPHOS capacity; Wust *et al.* 2015).

Table 2. Experimental models of sepsis.

ln vitro Models	
Cultured cells Blood cells Freshly isolated tissuc cells from septic animals	Do not reproduce trne sepsis (-) Suitable to study subcellular pathways in the particular cell types (+) Blood cells challenged by sepsis can be obtained from human patient<; (+)
ln vivo Models	
Peritonitis mode/s	
Cecal ligation and puncture Bacteria and fibrin clot implantation Colon ascendens stent peritonitis Cecal sllltťY injection Autologous feces inoculation	Polymicrobial (+) Pr esence ofinfection focus (+) Prolonged elevation of cytokines (+) Variable duration and severity (-) Approach true human sepsis (+) Poor control ofbacterial load (-) Surgical training requ ired (-) Resusc itation dcsirable (-)
J11travllscular mode/s	
Bacterial injection Endotox.in injection or infusion	Standardized, simple and reproducibl e (+) Dependent on the type of toxin, its dose, route of administration, host species (-) Differcnt from peritonitis models (-) Do not mimic hurnan sepsis (-) Strictly dependent on given PRR (-) High levels of inflammatory cytokines peak earlier (-) Usually fulminant disease (-) Suitable to study isolated effects of given endotoxin (+)

List of experimental models of sepsis with their advantages (+) and disadvantages (-). PRR - pattem recognition receptor.

Perspectives

Despite substantiaJ progress in understand ing of patbophysiology of sepsis and identification of a number of molecules that could be potentially beneficial in the treatment of sepsis, clinical trials designed to approve the use of these agents in the human medicine have been remarkably unsuccessful (Fink 2014). To improve the translational potential of animal experiments, several strategies have been suggested.

Modem omics technologies enable identification of multiple pathways potentially involved in the onset and progression of MOD in human septic patients (Evangelatos *et al.* 2018, Langley *et al.* 2013, Liu *et al.* 2014). Strategy of "reverse translation" based on identification of these pathways in bumans and their subsequent verification on appropriate animal model would help to avoid analysis and fruitless translation of phenomena that are strict ly species-specific (Efron *et al.* 2015).

The animal models are bcing continuously improved to better reproduce key features of human sepsis (Sto rtz *et al.* 2017). In rodents, use of cecal ligation and puncturc (CLP) resuscitatcd model is prefcrred to endotoxin and peritonitis models without resuscitation (Dejager *et al.* 2011). Experiments on small laboratory rodents will also enable evaluation of impact of age and various comorbidities on the pathophysiology and outcome of scpsis (Lofh1s *et al.* 2018, Miyaji *et al.* 2003). In contrast to vast majority of sepsis models in rodents, porcine experimental peritonitis is regular ly associated with hypcrdynamic pattern of circulation, increased oxygen delivery and unchanged systemic oxygen uptake, findin gs that mimic the course of hmnan disease (Benes *et al.* 2011 , Chvojka *et al.* 2008). Wider use of largcr animal species could be recommended in preclinical studies designed to approve the use of new pharmacological agents for the trcatment of sepsis.

To answer the question if mitochondrial dysfunction is a rcal determinant of induction/progression of sepsis-related organ dysfunction further intense researc h work is s till needed. Rcsearch in basic mitochondrial physiology requires more dctailed mapping of interspecies differeuces and organ-spccific features of oxygen utilization in addition to use of complex and standardized protocols evaluating mitochondrial respiration (Lemieux *et al.* 2017). Porcine models ot' sepsis allow repeated sampling that would belp to answer many questions conceming progrcssion of mitoc hondria l dysftmction and its impact on MOD (Matejovic *et al.* 2016).

Contlict of Interest

There is no conflict of interest.

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