Chronic Porcine Two-Hit Model with Hemorrhagic Shock and *Pseudomonas aeruginosa* Sepsis

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Abstract

Background: Sepsis is still a major cause of death despite well-developed therapeutical strategies such as antibiotics and supportive medication. The aim of this study was to characterize the long-term effects of a two-hit porcine sepsis model with a hemorrhagic shock as 'first hit' followed by a *Pseudomonas aeruginosa* infusion as 'second hit'. **Materials and Methods:** Twelve juvenile healthy pigs were anesthetized and hemodynamically monitored. The two-hit group (n = 6) underwent a hemorrhagic shock with a 50% reduction of the mean arterial pressure and/or cardiac index for 45 min, followed by resuscitation, while the control group (n = 6) received no pretreatment. All chronically catheterized conscious pigs were challenged with a *P. aeruginosa* infusion (1.6×10^7 CFU/kg/h for the first 24 h followed by 1.6×10^6 CFU/kg/h for the next 24 h) and observed for another 48 h. **Results:** The two-hit group showed the following significant differences to the control group: higher APACHE II scores prior to sepsis induction, increased persisting mean pulmonary arterial pressure (MPAP) and pulmonary vascular resistance index (PVRI) during bacterial challenge. In contrast, systemic vascular resistance (SVRI) was reduced at the end of the study. Throughout the observation period, the mean arterial pressure (MAP) was significantly reduced. **Conclusions:** The present study shows that the clinical course and hemodynamic effects of a *P. aeruginosa* sepsis will be aggravated by a preceding hemorrhagic shock during an oberservation period of 96 h. This two-hit model represents a valid, clinically relevant experimental protocol in sepsis research.

Introduction

Sepsis is a generalized response of the organism to an infectious insult and manifests itself as the systemic inflammatory response syndrome (SIRS). During the past decade, sepsis and related problems like acute respiratory distress syndrome (ARDS) have been the subject of intense investigations. As a result of numerous experimental studies, there is an improved understanding of the pathophysiological mechanisms underlying the organ dysfunction and metabolism in the septic state [1–4]. Despite the considerable progress in sepsis research, promising new therapeutic agents failed to show efficacy in clinical trials [5–8]. Preclinical trials using clinically inappropriate experimental models of sepsis or shock may have contributed to this dilemma. The single application of e.g. bacterial endotoxin treated with specific antagonist does not represent the clinical setting of human sepsis. There
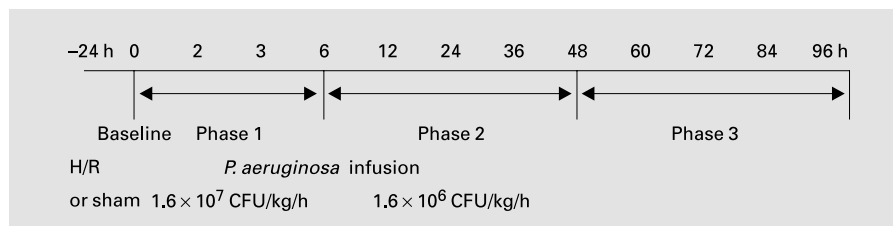
Fig. 1. Experimental timeline. (H/R = Hemorrhage/Resuscitation; Sham = Untreated).

are two basic principles of sepsis models: The 'one-hit model' postulates that massive tissue injury and shock produce intense systemic inflammation that results in early multiple organ failure (MOF). In human sepsis the systemic dysregulation is often associated with a priming insult, such as a prior episode of ischemia or trauma. Therefore a more common alternative is the 'two-hit model', which involves multiple sequential insults. Clinical observations of the SIRS response and MOF syndrome (MOFS) suggest that the immunological response to an initial 'hit' increases the organism's vulnerability to a second insult, like bacterial infections. This priming has been attributed in part to alterations in the immunological function of neutrophils and macrophages and includes not only maladaptive but also protective response resulting in tolerance. The tolerance to endotoxin and hemorrhage has been associated with alterations in the production and release of inflammatory mediators.

The aim of this study was to characterize and validate a chronic porcine two-hit sepsis model in the panel of preclinical sepsis research.

Materials and Methods

This study was designed in accordance with the German law of animal protection and has been approved by the Review Board for the Care of Animal Subjects at the Regierungspräsidium, Freiburg, Germany. The work reported in this article was carried out in accordance with the National Institutes of Health guidelines for the use of experimental animals.

Twelve German Landrace piglets (body weight 29 ± 3.9 kg, range 25–32 kg), were randomly assigned to the following groups: a control group receiving intravenous infusion of live *Pseudomonas aeruginosa* and a two-hit group with an initial hemorrhagic shock and resuscitation followed by bacterial challenge.

General Instrumentation
The animals were surgically prepared for this chronically instrumented study. After a 12-hour fasting period, the pigs were premedicated with intramuscular application of 10 mg/kg ketamine hydrochloride (Ketanest®, Parke-Davis, Freiburg, Germany) and 0.2 mg/kg flunitrazepam (Rohypnol®, Hoffmann-La Roche, Grenzach-

Wyhlen, Germany). Endotracheal intubation was performed after intravenous injection of 2–4 mg/kg propofol (Disoprivan®, Astra-Zeneca, Planckstadt, Germany). After endotracheal intubation the animals were mechanically ventilated with tidal volumes of 15 ml/kg and frequencies of 15 breaths/min. Antibiotic prophylaxis was administered (500 mg cefatiam hydrochloride, Spizef®, Grünenthal, Germany) before instrumentation. Catheters were placed in the carotid artery and external jugular vein for administration of fluids and for monitoring hemodynamics. A Swan-Ganz® thermal dilution catheter (110 cm, 7 Fr, Baxter, Unterschleißheim, BRD) was positioned through the external jugular vein into the pulmonary artery. In all animals, the indwelling jugular venous catheters and arterial catheters were left in situ, tunneled to the exterior and sutured at the skin. After wound closure, the animals were weaned from ventilation and returned to the cage.

Shock Protocol
Within 30–45 min 35–40% of the estimated blood volume was withdrawn through the arterial catheter. Shed blood was stored in sterile blood bags (Compoflex®, NPBI, Netherlands). After a 45–60-min shock period, resuscitation was initiated with plasma expander (colloid fluid, HAES 6%®, Fresenius Kabi, Germany) followed by cristalloid fluid (Jonosteril®, Fresenius Kabi). The total fluid volume was two times the shed blood volume and was administered within the first hour of resuscitation. Resuscitation also included shed blood. The effects of anesthesia, intubation, cannulation, etc. were controlled by the control group (sham group) which was instrumented and treated with antibiotics, but not subjected to hemorrhage. During the observation period of 96 h the animals were monitored according to the protocol under sedation.

Experimental Protocol
The experiment was performed in awake pigs. The animals were divided into the following groups: The control group (n = 6) received a *P. aeruginosa* infusion 24 h after surgical catheter implantation, without a preceding hemorrhagic shock. The two-hit group (n = 6) underwent a 45-min hemorrhagic shock period followed by resuscitation before bacterial challenge. After baseline measurements, all animals received a continuous infusion of live *P. aeruginosa* bacteria at a dose of 1.6×10^7 colony-forming units (CFU)/kg/h for the first 24 h. For the following 24 h the *P. aeruginosa* infusion was reduced at a dose of 1.6×10^6 CFU/kg/h. The infusion rate of Ringer lactate was adjusted to keep the central venous pressure at baseline levels (± 2 mm Hg). After bacterial challenge the animals were monitored for 48 h. The observation period was divided into three phases: phase 1 (2–6 h), phase 2 (6–48 h) and phase 3 (48–96 h). The baseline values (Status S1) of all parameters were documented before bacterial challenge. The general experimental timeline is shown in figure 1.

Bacterial Preparation and Quantitative Cultures

In this study live *P. aeruginosa* was used for induction of sepsis. *P. aeruginosa* International Antigenic Typing Scheme serotype 1 (ATCC 33348) was obtained from K.D. Hungerer, Behringwerke Marburg, Germany. Aliquots of 1 ml (5×10^8 CFU of log-phase-grown bacteria) were stored at $-70°C$ in 50% glycerol. 48 h before the experiments the bacteria were prepared from these frozen stock culture. After being thawed, a tip of the stock culture was inoculated on an agar plate for 24 h at 37°C.

Bacteria were grown at 37°C with vigorous shaking. For challenge experiments, the bacteria were harvested at mid log phase, washed three times with sterile phosphate-buffered saline (Life Technologies Inc., Eggenstein, Germany) and adjusted to the required concentration with 0.9% saline. The bacterial density in cells per milliliter was calculated from optical density at 546 nm. Serial 10-fold dilutions were plated on blood agar plates, and the CFU were counted after overnight incubation. The adjusted bacterial suspension was then placed in a 50-ml syringe (Perfusor syringe, Braun Melsungen, Germany). During intravenous application the infusion system was stored at 4°C. Fresh bacterial infusions were prepared every 6 h.

To assess bacteremia, arterial blood (200 µl) was cultured on tryptic soy agar with 5% sheep blood plates. To assess the quantity of bacterial tissue retention the organs were harvested and homogenized. Bacterial colonies were counted after incubation for 24 h at 37°C. The quantity of bacteria was determined by multiplying counted colonies by the dilution rate.

Data Collection

The following parameters were monitored at baseline (before bacterial challenge), and 2, 3, 6, 12, 24, 48, 72 and 96 h after *Pseudomonas* challenge: modified APACHE II scoring, body temperature, heart rate, respiratory rate, mean arterial pressure (MAP), central venous pressure, mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure and cardiac output. Hemodynamic parameters were calculated from the measured variables (cardiac index = cardiac output/body weight, systemic vascular resistance index (SVRI) and pulmonary vascular resistance index (PVRI)).

Hematocrit, blood gases (partial pressure of oxygen PaO_2, partial pressure of carbon dioxide $PaCO_2$, arterial oxygen saturation SaO_2, pH, base excess), and blood electrolytes (Na^+, K^+, Cl^-, Ca^{++}, glucose) were recorded at these intervals on a Radiometer ABL 500® (Radiometer Copenhagen, Denmark). Blood samples were drawn at regular intervals for differential cell counts, cytokine assay for tumor necrosis factor α (TNFα), IL-10 (ACM Biotech, Regensburg, Germany), IL-6 (R&D Systems, Wiesbaden, Germany) and blood cultures. After an observation period of 96 h the animals were killed, lung, liver, kidney and heart were fixed in formalin for histopathology as well as homogenized for tissue retention.

Statistics

Parameters were compared over the time course using an analysis of variance (ANOVA) for repeated measures. For within-group differences multiple comparisons were made with Bonferroni adjustments for the effects of treatment at specific times, and for the effect of time in specific group with baseline as a control (significant difference: * = control group, # = two-hit group). Between-group differences were analyzed using 2-way ANOVA with repeated measures followed by Student's t test (§ = differences between control and two-hit group). Data were presented as mean values ± standard deviation (SD). p Values <0.05 were judged as significant.
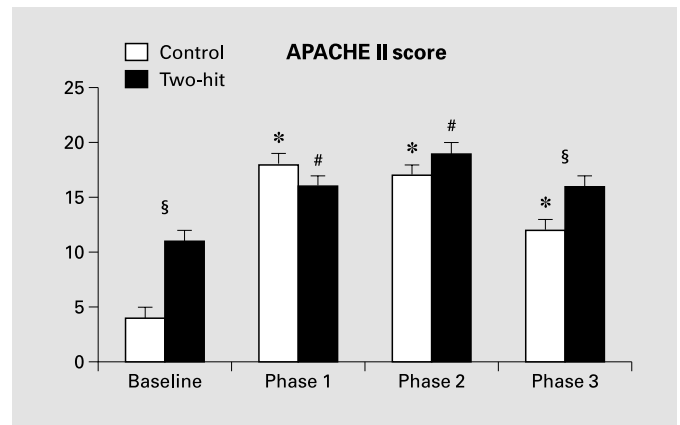
Fig. 2. APACHE II Score. Significantly higher scoring of the two-hit group before bacterial challenge and at the end of the study compared with the control group.

Results

In our experimental sepsis models all animals survived the 96-hour observation period and both groups produced a hyperdynamic sepsis with elevated cardiac index, low SVRI, systemic hypotension and increased pulmonary arterial pressure during *P. aeruginosa* infusion.

The APACHE II score of the two-hit group was significantly higher prior to bacterial infusion (control 4 ± 2, two-hit 11 ± 5) and in phase 3 (control 12 ± 2, two-hit 16 ± 3) compared with the control animals. During bacterial challenge (phases 1 and 2) the APACHE II scores of both groups were significantly increased compared with baseline values (fig. 2). The body temperature was initially reduced in the two-hit group compared with the control group (data not shown).

Changes in Hemodynamic and Blood Gas Parameters

The MAP of the two-hit group was significantly reduced in all 3 phases compared with the control group. In phase 2 the MAP of both groups was significantly reduced (control 101 ± 10, two-hit 76 ± 17) (fig. 3).

The baseline MPAP of the animals pretreated with a hemorrhagic shock was not increased compared with the sham-operated animals. Within 2 h of bacterial infusion the MPAP increased in both groups and peaked significantly in phase 1 (control 14 ± 2, two-hit 23 ± 11). The MPAP decreased with reduction of the bacterial dose. The between-group difference in phases 2 and 3 was significant. The higher MPAP of the two-hit group persisted throughout the 96 h (fig. 4).
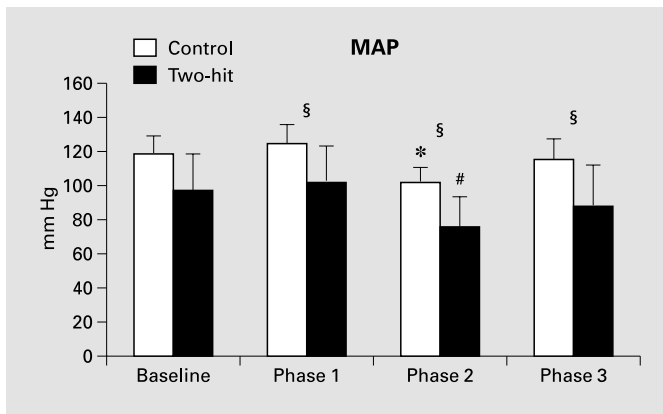
Fig. 3. Mean arterial pressure. Suppression during the observation period of 96 h. Significant difference between control and two-hit group.
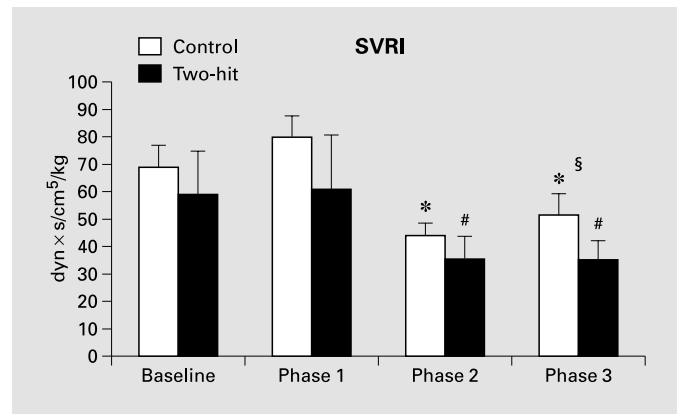

Fig. 5. Systemic vascular resistance index. Suppression in phase 3. Significant difference between control and two-hit group.
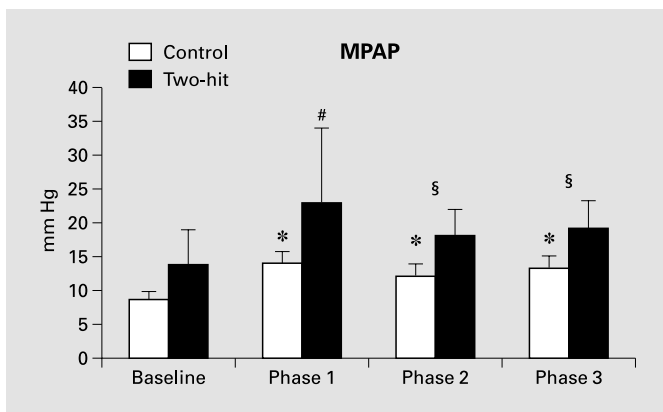

Fig. 4. Mean pulmonary arterial pressure. Elevated at the end of the study. Significant difference between control and two-hit group.
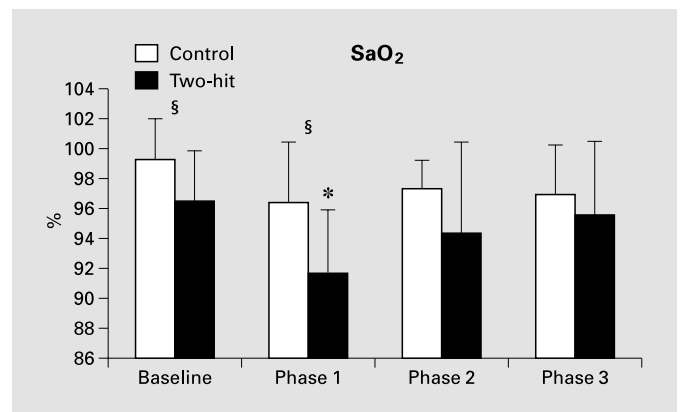

Fig. 6. Pulmonary vascular resistance index. Elevated in phase 2. Significant difference between control and two-hit group.

The SVRI of both groups was significantly reduced in phases 2 and 3 compared with baseline levels. In phase 3 the SVRI of the shocked animals was significantly reduced (35 ± 7) compared with control animals (51 ± 8) (fig. 5). The PVRI was not different before bacterial challenge and increased significantly (control 7 ± 1, two-hit 11 ± 5) during high-dose *Pseudomonas* infusion (phase 1) in both groups. The two-hit group showed a significantly increased PVRI in phase 2 (control 4 ± 1, two-hit 6 ± 2) (fig. 6).

There were no significant between-group differences with regard to hemodynamic measurements including central venous pressure, pulmonary capillary wedge pressure or cardiac output (data not shown).

Blood gas analysis (fig. 7) of the two-hit animals showed a significant reduction of the arterial SaO_2 before and during the first 24 h of *Pseudomonas* infusion compared with control animals (control 99.3 ± 2.6 and 96.5 ± 4 vs. two-hit 96.5 ± 3.3 and 91.7 ± 4.3).

Hematologic Changes and Cytokine Levels

There were no differences in lymphocyte and polymorphonuclear neutrophil (PMN) counts between control and shock group. A transient thrombocytopenia occurred in both groups in phase 2. Hematocrit did not change in any group.

The time course of the plasma cytokine induction during the whole observation period reveals a similar profile
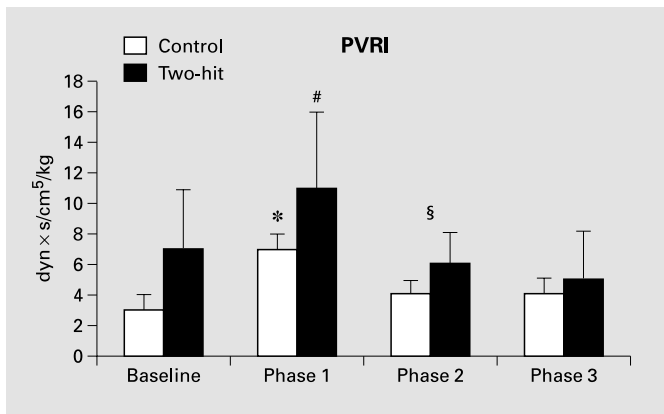
Fig. 7. Arterial SaO$_2$. Suppression before and during high dose *P. aerugionsa* infusion (phase 1). Significant difference between control and two-hit group.
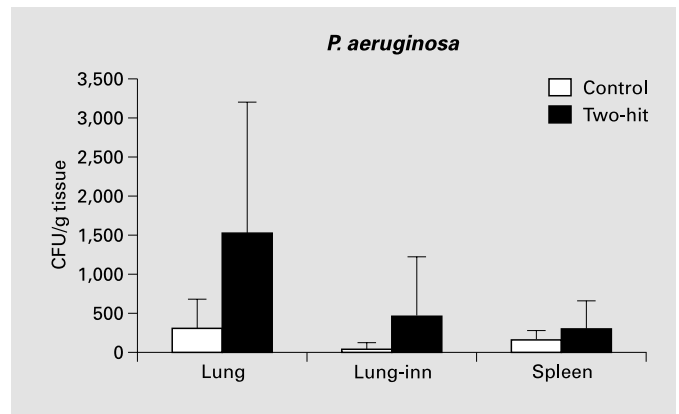

Fig. 9 Tissue retention. Higher *P. aeruginosa* contamination of the lung and lymph nodes of the lung in the two-hit group compared with the control group.
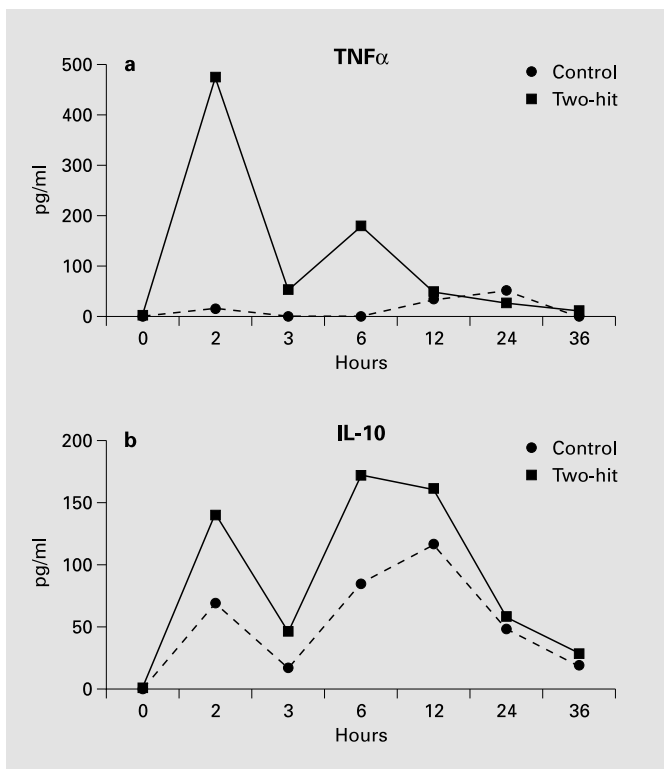

Fig. 8. Cytokine profile. **a** TNFα. TNFα-peak within the first 2 h of *P. aeruginosa* infusion in the two-hit group. No elevated TNFα level in the control group. **b** IL-10. Double peak (2 and 12 h of bacterial challenge) in the control and two-hit group.

between both groups with slightly higher levels in the two-hit group. Plasma TNFα was markedly increased in the two-hit group 2 h after bacterial challenge (fig. 8a). The proinflammatory cytokine IL-6 reached a maximum after 6 h (not shown), followed by a IL-10 double-peak (2 and 12 h after bacterial challenge) (fig. 8b).

Histological Findings/Tissue Retention

Lung injury was attenuated after pretreatment with hemorrhagic shock. The lungs were characterized by a moderate to marked interstitial pneumonia, intralobular and perivascular edema, and fibrin deposition in capillaries and alveoli. Livers were congested with mild biliary stasis. Kidneys were mostly normal with occasional mild congestion or acute tubular necrosis, but there was no detectable difference in the other categories (data not shown).

The bacterial contamination of lung und lymph nodes of the lung was significantly higher in the two-hit group due to hemorrhage-induced impairment in antibacterial host defense of the lung (fig. 9).

In summary, hemorrhagic shock caused a higher APACHE II score, significantly reduced MAP and arterial SaO$_2$ prior to bacterial challenge. A significant pulmonary hypertension developed within 2 h of bacterial challenge and persisted throughout the study in the two-hit group, while the MPAP of the control group reached baseline values at the end of the study. After 48 h of bacterial challenge the measured variables of the control groups returned to baseline values, while the systemic hypotension, elevated pulmonary arterial pressure and low systemic vascular resistance index in the two-hit group per-

sisted throughout the 96-hour observation period. In conclusion, the two-hit group demonstrated an increased susceptibility to sepsis with pronounced clinical signs of a severe sepsis.

Discussion

Based on our previously published data of a porcine *P. aeruginosa* sepsis [9] we modified a clinically relevant two-hit sepsis model with a hemorrhagic shock prior to a low-dose *P. aeruginosa* infusion for 48 h. Compared with the one-hit control group, the pretreated animals showed significantly aggravated and persisting organ dysfunctions, especially pulmonary failure.

Deitch et al. [10] reviewed and discussed several experimental sepsis and hemorrhagic shock models. They compared basic principles of experimental studies with clinical trials and recommended that a relevant model for human disease should consider a natural route of infection, virulent bacteria, hemorrhagic shock due to uncontrolled hemorrhage, no pretreatment, adjuvant therapy, different insults, natural progression of the disease, clinically relevant endpoints and an optimal active care.

The aggravated lung injury (MPAP, PVRI, SaO$_2$) of our two-hit group was induced by the hemorrhagic shock as the first hit. This priming insult leads to the induction of an extensive impairment on cellular immune responses and initial cytokine downregulation [11, 12], affecting lung innate immunity [13]. These initial immunological events sensitize the host against the subsequent infectious insult as it is confirmed by Cue et al. [14], who state that the hemorrhagic shock is a major determinant of the risk of infection and respiratory immunosuppression. Our data on the selective induction of TNFα after the second hit confirm the impact of the first hit for a systemic immune dysregulation. On the other hand, the effect of an initial hemorrhagic shock also involves macrophage dysfunction. Blood withdrawal and subsequent fluid substitution induce a downregulation of complement and Fc receptors on macrophages, leading to an impaired phagocytic response and consequently to an increased susceptibility to bacterial infection [15, 16].

Microvascular changes are one explanation of the 'two-hit' theory of multiple organ failure [17–20]. Gelfand et al. [21] support the concept that the hemorraghic shock accounts for the septic complication, since they did not find any significant bacterial translocation within 6 h of hemorrhagic shock in the pig. In contrast, Morales et al [22] published a porcine two-hit model with *E. coli* challenge and hemorrhagic shock. They found a bacterial translocation in the late phase of the shock.

Fan et al. [23] demonstrated in a two-hit model with preceding shock and low-dose intratracheal LPS administration in the rat a priming effect of a hemorrhagic shock for increased lung injury and PMN sequestration. PMN are activated after resuscitation from traumatic shock and produce an endothelial injury that may increase the vulnerability to septic challenge [24]. Another study of Fan et al. [25] demonstrated that ischemia/reperfusion initiated by resuscitated hemorrhagic shock primes the lungs for a procoagulant response after a second inflammatory stimulus i.e. low-dose intratracheal endotoxin. Our histological analyses revealed a marked attenuated lung injury as well as a significantly higher bacterial tissue contamination that might contribute to pulmonary failure.

In conclusion, our chronic two-hit sepsis study consisting of a hemorrhagic shock and the application of low-dose *P. aeruginosa* might be a clinically relevant experimental model for the development of new therapeutic strategies.

Acknowledgments

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