

## AN IMPROVED MODEL OF SEVERE SEPSIS IN PIGS

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## REZUMAT

**Obiectiv:** Dezvoltarea unui model de studiu al sepsisului pe animalul mare de laborator prin inducerea peritonitei fecale, cu reproducerea caracteristicilor macrohemodinamice, microcirculatorii si inflamatorii, obiectivate in sepsa umana incipienta. **Material si metode:** Porcii pitici anesteziati au fost supusi peritonitei fecale (n = 9; 0,5 g/kg i.p., autofecale) sau operatiei-control (ser fiziologic i.p., n = 6). Gazimetria a fost monitorizata hemodinamic invaziv la 15-24 ore postchirurgicale. Microcirculatia sublinguala a fost exprimata prin modificarile vitezei eritrocitare (MVE) (prin imagistica spectrala a polarizarii octogonale), in timp ce intervalul PCO<sub>2</sub> intestinal a fost masurat prin tonometrie indirecta. Nivelele plasmatiche ale proteinei HMGB<sub>1</sub> si oxidului nitric/nitrat (NOx) au fost determinate din probe de sange venos. **Rezultate:** Presiunea arteriala medie a scazut treptat sub 70 mm Hg la animalele cu sepsis, in timp ce frecventa cardiaca si debitul cardiac au crescut constant. In ciuda reactiilor hiperdinamice, MVE a scazut, in timp ce intervalul PCO<sub>2</sub> a crescut semnificativ, in comparatie cu grupul control. Concentratiile plasmatiche ale NOx si HMGB<sub>1</sub> au fost semnificativ crescute la 6-16 ore de la debutul peritonitei. **Concluzii:** Raportam dezvoltarea unui nou model experimental de studiu al sepsisului prin inducerea peritonitei fecale la animalul de laborator. Datele obtinute in vivo sugereaza ca acest model experimental este relevant clinic si poate avea un rol inovator in dezvoltarea terapiilor anti-sepsa.

**Cuvinte cheie:** sepsa, circulatie hemodinamica, microcirculatie sublinguala

## ABSTRACT

**Objective:** Our aim was to develop a large animal model of sepsis, induced by fecal peritonitis, which reproduces the characteristic macrohemodynamic, microcirculatory and inflammatory changes seen in early human sepsis. **Material and methods:** Anesthetized minipigs were subjected to fecal peritonitis (n = 9; 0.5 g/kg i.p. autofeces) or sham-operation (i.p. saline, n = 6). Invasive hemodynamic monitoring was started with regular blood gas analyses between the 15-24 hr of the insult. Sublingual microcirculation was characterized by red blood cell velocity (RBCV) changes (with orthogonal polarization spectral imaging), while the intestinal PCO<sub>2</sub> gap was measured by indirect tonometry. The plasma levels of high mobility group box protein 1 (HMGB<sub>1</sub>) and nitrite/nitrate (NOx) were determined from venous blood samples. **Results:** The mean arterial pressure gradually decreased below 70 mm Hg in septic animals, while the heart rate and cardiac output increased constantly. In spite of the hyperdynamic reaction the sublingual RBCV decreased, while intestinal PCO<sub>2</sub> gap increased significantly as compared with the control group. The NOx and HMGB<sub>1</sub> plasma concentrations were significantly elevated between 6-16 hr of peritonitis. **Conclusion:** We report on the introduction of a new animal model fecal peritonitis-induced sepsis. The in vivo data suggest that this experimental model is of clinical relevance and may play useful roles in the development of novel, sepsis-related therapies.

**Key Words:** sepsis, hyperdynamic circulation, sublingual microcirculation, high mobility group box protein 1

## INTRODUCTION

Sepsis remains a leading cause of mortality in intensive care units (ICUs), without specific therapeutic options. Basic medical research utilizing animal models has provided a greater understanding

of its underlying mechanisms, and today the involvement of canonical inflammatory pathways such as the activation of the complement system, leukocytes, lipid mediators and adhesions molecules has been relatively well defined. Nevertheless, the parallel development of clinical treatment strategies did not progress significantly during the last decades. Since similarities between the human and animal responses to septic insults should help to understand the key factors of therapeutic control, the lack of success clearly demonstrates that the currently used in vivo models do not completely mimic human septic conditions.<sup>1,2</sup> By definition, a model is a tool via which to understand or describe a system or phenomenon, and the "goodness of fit" through usability testing is its most important characteristic. This suggests that a model with improved clinical relevance may play a

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more useful role in the development of novel, sepsis-related therapies.<sup>3</sup>

Our goal was to narrow the gap between experimental and human sepsis by putting much greater emphasis on the reproduction of ICU scenarios and the consequences of intensive treatment. By this approach, not only the septic insult-induced changes, but other, therapy-caused alterations could be considered, similarly to those seen in clinical conditions. To this aim a standardized porcine model of protracted peritonitis was created using intraperitoneal autologous fecal inoculum and complex resuscitation strategies. This communication reports on the most important specific features of the setup, including reproducible macrohemodynamic and microcirculatory variables, and characteristic biochemical data.

## **MATERIAL AND METHODS**

The study protocol was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

The experiments were performed on Vietnamese minipigs of both sexes (average weight  $23 \pm 3$  kg) which were underwent a 16-hr preoperative fasting with water ad libitum; the animals were randomly allocated into control (sham-operated;  $n = 6$ ) and septic groups ( $n = 9$ ). Anesthesia was induced with an intramuscular injection of a mixture of ketamine (20 mg/kg) and xylazine (2 mg/kg) and maintained with a continuous infusion of propofol (6 mg/kg/hr iv). An endotracheal tube was inserted and the animals were ventilated mechanically with room air (Harvard Apparatus, South Natick, MA, U.S.A.) The tidal volume was set at  $12 \pm 2$  mL/kg, and the respiratory rate was adjusted to maintain the end-tidal carbon dioxide pressure (controlled by capnometry) and the partial arterial carbon dioxide pressure in the range of 35-45 mm Hg (4.6-5.9 kPa). The adequacy of the depth of anesthesia was assessed by monitoring the jaw tone regularly. A central venous catheter with three lumina (7 F; Edwards Lifesciences LLC, Irvine, U.S.A) was introduced into the jugular vein using aseptic surgical technique, for blood sampling and for fluid administration, respectively.

Sepsis was induced with an intraperitoneal injection of autofaeces mixture into the abdominal cavity (0.5 g/kg faeces in 200 ml saline and cultivated at 38°C through 6 hrs until the induction of peritonitis). Control animals were treated with 200 ml of sterile saline in the same manner. Thereafter, a single nalbuphine (0.5 mg/kg iv) injection was used

for postoperative analgesia and the sedated animals were brought back to their cages.

Invasive haemodynamic monitoring was started 15 hr later. A transpulmonary thermodilution catheter (PiCCO, PULSION Medical Systems AG, Munich, Germany) was placed in the femoral artery and a pulmonary artery catheter (PV2057 VoLEF Catheter, PULSION Medical Systems AG, Munich, Germany) was introduced via the femoral vein by tracing the pressure signals. A midline laparotomy was performed and a tonometric probe was introduced into the small intestine through a small incision to record mucosal  $p\text{CO}_2$  changes. Thereafter the most commonly used volume therapies were applied in the septic group of animals. Infusion of crystalloid-colloid fluid combinations [lactated Ringer's solution at a rate of 10 ml/kg/hr; hydroxyethyl starch in a dose of 5 ml/kg/hr (Voluven 6%; 130 kDa/0.4; Fresenius Kabi Deutschland GmbH, Homburg, Germany)] was started for supporting intravascular volume through 4 hrs (between 16<sup>th</sup> and 20<sup>th</sup> hour of the experiments). At the final part of the study period vasopressor therapy (0.015  $\mu\text{g}/\text{kg}/\text{hr}$  norepinephrine in 20 ml saline iv) was started if the MAP decreased under 65 mmHg to avoid the kidney failure (the pressor treatment was necessary in 4 out of 9 animals). The sham-operated group was infused with crystalloid solution (lactated Ringer's solution) during this time at a rate of 10 ml/kg/hr.

### **Hemodynamic measurements**

Mean arterial pressure (MAP) and cardiac output were registered by PiCCO monitor, while central venous pressure (CVP) and pulmonary artery pressure (PAP) signals were monitored continuously with a computerized data-acquisition system (SPELL Haemosys; Experimetria Ltd., Budapest, Hungary). The systemic vascular resistance (SVR) was calculated via the standard formula  $\text{TPR} = (\text{MAP} - \text{CVP})/\text{cardiac output}$ .

### **$p\text{CO}_2$ gap measurements**

A difference between local tissue and arterial  $p\text{CO}_2$  ( $\text{paCO}_2$ ) levels is a sensitive parameter with which to evaluate the effectiveness of therapy aimed at counteracting a microcirculatory dysfunction in the gastrointestinal (GI) tract.<sup>4</sup> A silastic balloon-free tonometric probe (Tonosoft Medical Technical and R&D Ltd., Hungary) was introduced through a small enterotomy into the intestinal lumen to monitor intramucosal  $p\text{CO}_2$  levels by capnometry.<sup>5</sup> For calculation of the  $p\text{CO}_2$  gap values, simultaneously taken  $\text{paCO}_2$  levels were subtracted from the tonometric  $p\text{CO}_2$  levels. Arterial blood samples were taken regularly, and blood-gas parameters were

measured with a blood-gas analyzer (Cobas b121, Roche, Austria).

### Intravital videomicroscopy of the microcirculation

The intravital orthogonal polarization spectral (OPS) imaging technique (Cytoscan A/R, Cytometrics, Philadelphia, Pennsylvania, USA) was used for non-invasive visualization of the sublingual microcirculation.<sup>6</sup> The OPS method makes use of reflected polarized light, which allows noninvasive imaging of the microcirculation on the surface of solid organs without the need for fluorescence contrast enhancement. In brief, linearly polarized light is scattered in the tissue and serves as a virtual light source. Images are obtained at 548 nm wavelength, which is the isobestic point for oxy- and deoxyhemoglobin. In this way red blood cells in the microcirculation appear in black on the white background of the surrounded tissue. A 10x objective was placed onto the surface of the sublingual area, and microscopic images were recorded with an S-VHS video recorder (Panasonic AG-TL 700, Matsushita Electric Ind. Co. Ltd, Osaka, Japan). Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Red blood cell velocity (RBCV,  $\mu\text{m/s}$ ) changes in the postcapillary venules were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). All microcirculatory evaluations were performed by the same investigator.

### Plasma nitrite/nitrate level measurements

The levels of plasma nitrite/nitrate ( $\text{NO}_x$ ), stable end-products of nitric oxide (NO), were measured by the Griess reaction. The assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a colored azo compound detected spectrophotometrically at 540 nm.<sup>7</sup>

### High mobility group box protein 1 measurements in plasma

Two-ml blood samples were drawn from the jugular vein into chilled polypropylene tubes containing EDTA ( $1 \text{ mg ml}^{-1}$ ) at baseline, the 6<sup>th</sup>, 16<sup>th</sup> hour and at the end of the observation period (24<sup>th</sup>). The blood samples were centrifuged at 1200g for 10 min at 4°C. The plasma samples were then collected and stored at -70°C until assay. Plasma concentration of high mobility group box protein 1 ( $\text{HMGB}_1$ ) was measured by a commercially available  $\text{HMGB}_1$  ELISA kits (Shino-Test Corporation, Kanagawa, Japan).

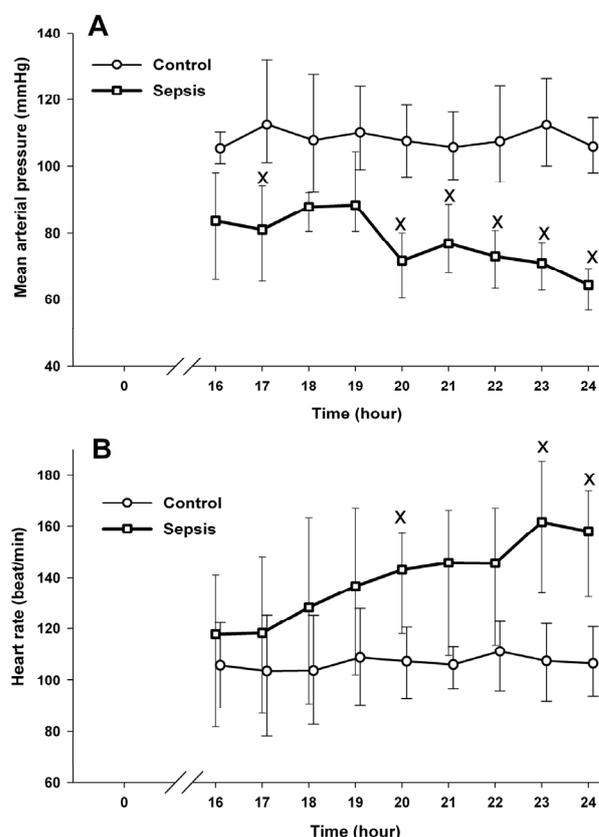
### Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel

Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline (time 0), or from the beginning of the invasive monitoring (the 16<sup>th</sup> hr of the observation period) for each group were assessed by Dunn's method, and differences between groups were analyzed with Mann-Whitney test. In the Figures, median values and 75<sup>th</sup> and 25<sup>th</sup> percentiles are given. P values < 0.05 were considered significant.

## RESULTS

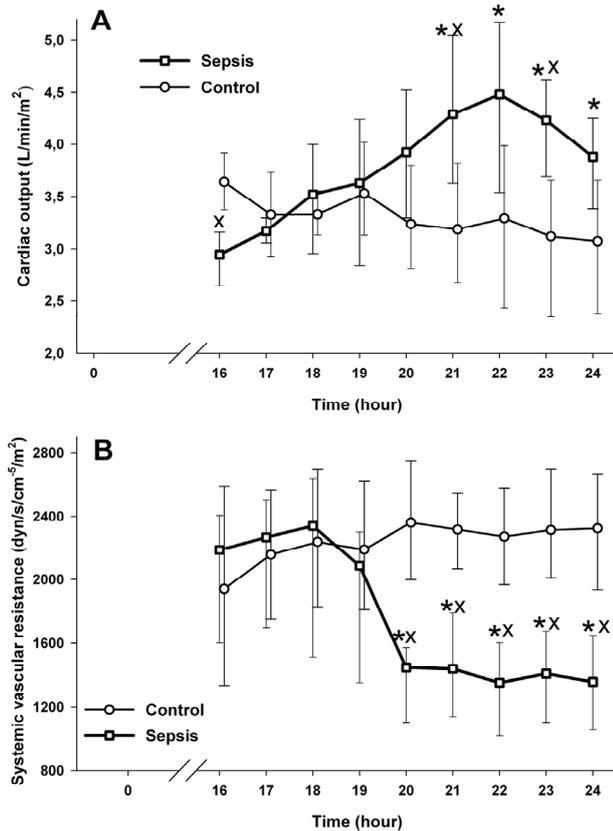
In the control group, there were no significant hemodynamic changes as compared with the baseline values, and the plasma mediator levels did not change significantly during the observation period.



**Figure 1.** Changes in the mean arterial pressure (A) and the heart rate (B) in the control and septic groups. The plots demonstrate the median values and the 25<sup>th</sup> (lower whisker) and 75<sup>th</sup> (upper whisker) percentiles, \* $p < 0.05$  within groups vs. baseline values, x  $p < 0.05$  between groups vs. control group values.

There were statistical significant differences in hemodynamics between control and septic groups. The MAP gradually decreased below 70 mmHg in septic animals (Fig. 1A), while the heart rate increased constantly and exceeded the control values significantly (Fig. 1B). In the septic group, the cardiac index was

remarkably elevated and surpassed significantly the control level from the 20<sup>th</sup> hr. (Fig. 2A) These changes resulted in dramatic decreases of the SVR. In the septic group the SVR started to decrease at the 18<sup>th</sup> hr of the experiments and reached the deeper point at 20<sup>th</sup> hr and then the values were kept at this low level until the end of the study. (Fig. 2B)



**Figure 2.** Changes in cardiac output (A) and the systemic vascular resistance (B) in the control and septic groups. The plots demonstrate the median values and the 25<sup>th</sup> (lower whisker) and 75<sup>th</sup> (upper whisker) percentiles, \*p<0.05 within groups vs baseline values, x p<0.05 between groups vs control group values.

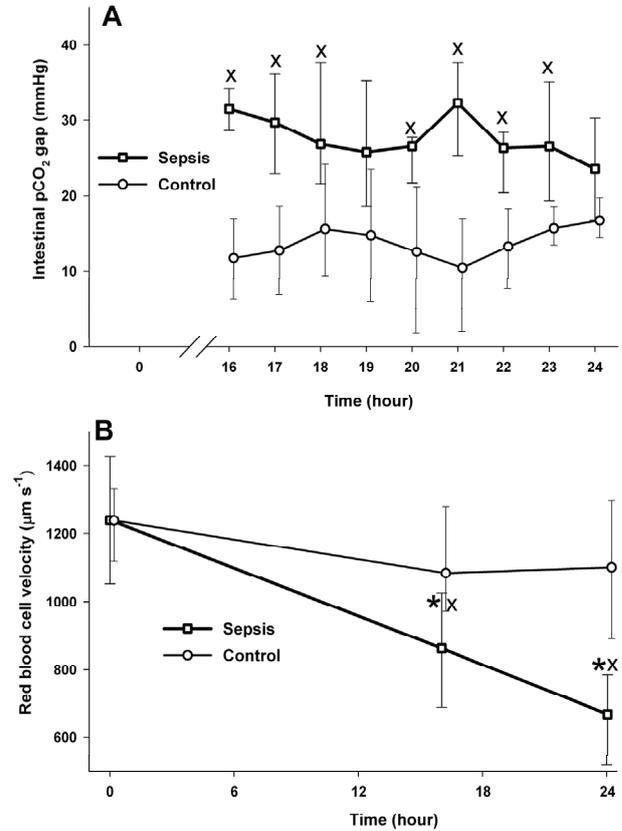
The pCO<sub>2</sub> gap is the difference between the local tissue and the arterial pCO<sub>2</sub>, and a reliable index of local tissue perfusion. The pCO<sub>2</sub> gap of the small intestine in the septic group increased significantly at the beginning of the invasive monitoring (from the 16<sup>th</sup> hr of sepsis), and remained significantly higher than that for the sham-operated control group. (Fig. 3A)

The analysis of the sublingual microcirculation, which represents the peripheral microperfusion status, revealed a gradually decreasing and statistically significantly lower red blood cell velocity in the septic group as compared to both baseline values and the control group, from the 16<sup>th</sup> hr of the experiment. (Fig. 3B)

The NOx concentration in the plasma gives an estimate of the changes in NO production. Sepsis induction resulted in a statistically significant increase

in NOx level as compared with the baseline values and to the control group. (Fig. 4A)

The plasma HMGB<sub>1</sub> concentration gradually increased approx. 5-fold by 16 hr after of the induction of sepsis and remained significantly higher than in the control group up to 24 hr in the observation period. (Fig. 4B)

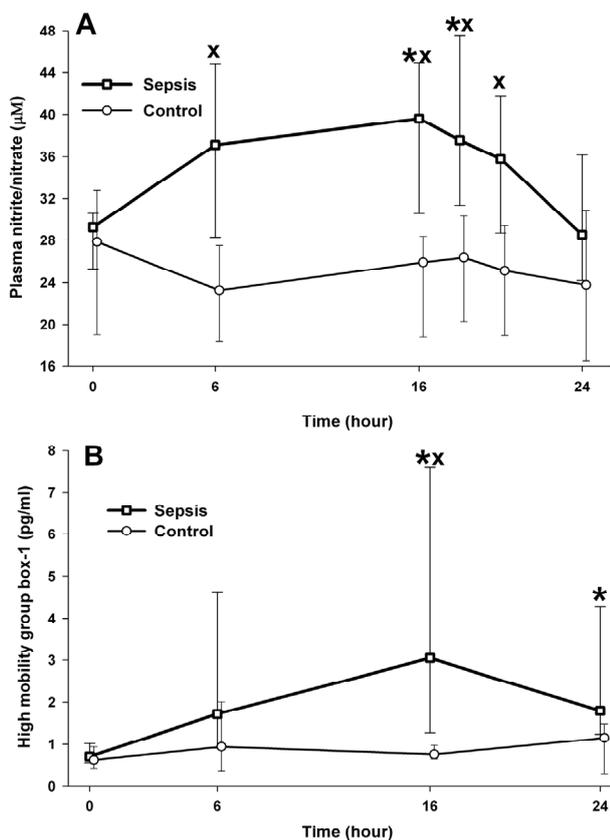


**Figure 3.** Changes in the intestinal pCO<sub>2</sub> gap (A) and the red blood cell velocity of the sublingual microcirculation (B) in the control and septic groups. The plots demonstrate the median values and the 25<sup>th</sup> (lower whisker) and 75<sup>th</sup> (upper whisker) percentiles, \*p<0.05 within groups vs baseline values, x p<0.05 between groups vs control group values.

## DISCUSSIONS

Several animal models try to replicate the signs and laboratory findings seen in human sepsis, where systemic hemodynamics evolves from an early hyperdynamic (“warm shock”) state to a late hypodynamic (“cold shock”) state. Such models include intraperitoneally, intravenously or intrapulmonally-administered endotoxin (lipopolysaccharide) or live bacteria.<sup>8,9</sup> Cecal ligation and perforation or induction of bacterial peritonitis with fecal inoculum are also frequently used.<sup>10,11</sup>

However, it should be stressed that hemodynamic and microcirculatory measurements together with blood sample collections are difficult to perform in rodents, and short-term, hypodynamic models have limited clinical relevance.<sup>2</sup>



**Figure 4.** Changes in the plasma levels of nitrite/nitrate (A) and the high mobility group box protein 1 (B) in the control and septic groups. The plots demonstrate the median values and the 25<sup>th</sup> (lower whisker) and 75<sup>th</sup> (upper whisker) percentiles, \* $p < 0.05$  within groups vs baseline values,  $\times p < 0.05$  between groups vs control group values.

These are all characterized by initial hypotension and low cardiac output, in contrast to the hyperdynamic circulation commonly seen in patients with septic shock. Furthermore, rodent reactions are markedly different from human with respect to the immune response to sepsis or the tissue antioxidative capacity and susceptibility to oxidative stress.<sup>12,13</sup>

Indeed, several therapies proposed based on promising results obtained by these approaches could not be brought to the clinic, and many reviews have shown that the failure to translate basic science results from animals to humans has been mainly attributed to inappropriate animal models that do not fully mimic human sepsis.

In our study we switched to larger animals, similarly to Barth et al., who used a comparable, long-term porcine fecal peritonitis model, in which the hyperdynamic circulatory reaction was present between 12–18 hrs after sepsis induction.<sup>14</sup> However, we did not employ 24 hrs anesthesia, and in contrast to the Barth's model, the hemodynamic monitoring started later, 15 hr after the insult.

In this setup the intra-abdominal septic insult could be accurately standardized with the pre-defined amount

fecal inoculum, and consequently, the inflammatory-hemodynamic responses were reproducible.

It has been demonstrated in ICU patients that normalization of hemodynamic status in itself is insufficient to prevent the postoperative complications, since existing microcirculatory disturbances can lead to nutritive insufficiency and multiorgan failure in spite of seemingly adequate macrocirculation.<sup>15</sup>

Non-invasive imaging techniques, such as OPS and its successor Sidestream Dark Field (SDF) imaging are optical techniques allowing assessment of the microperfusion in case of solid organs, covered with thin epithelial layer, as the sublingual mucosal surfaces.<sup>6,15</sup> Indeed, it has been demonstrated in humans that improvement in the sublingual microvascular perfusion, as early as 24 hrs after the onset of shock, can be a good predictor of ICU mortality.<sup>16</sup>

Our study demonstrated different patterns of microvascular alterations between septic and control animals in both intestinal and sublingual regions, and these data clearly indicated the presence of substantial intestinal and peripheral hypoxia in spite of the hyperdynamic macrocirculation.

The microcirculatory reaction could be a consequence of the altered synthesis of NO and proinflammatory cytokines. It is generally accepted that NO produced by constitutive NOS (cNOS), including nNOS and eNOS, are important homeostatic regulators of numerous important physiological functions.

In contrast, inducible NO synthase (iNOS) is produced by inflammatory cells, induced by various stimuli such as inflammatory cytokines and bacterial endotoxin, and plays an important role in inflammation.<sup>17</sup> Thus, a long lasting elevation of the NOx level of plasma may be considered a hallmark of inflammation in the present study.

A systemic response to infection is characterised by early-phase inflammatory cytokines, such as tumor necrosis factor (TNF)-alpha or interleukin (IL)-1 beta. In this respect the high-mobility group box-1 protein (HMGB<sub>1</sub>) has recently been shown to be a late-phase mediator of sepsis with close correlation with the severity of the process.<sup>18,19</sup> In our study the plasma HMGB<sub>1</sub> levels peaked 16 hr after the start of septic reaction and remained significantly elevated.

HMGB<sub>1</sub> is secreted by activated monocytes or macrophages, and is released by necrotic or damaged cells. Extracellular HMGB<sub>1</sub> mediates cell-to-cell signalling, provokes the production of inflammatory cytokines and subsequently activated cytokines can induce further release of HMGB<sub>1</sub> into the extracellular space.<sup>20</sup>

## **CONCLUSION**

The micro- and macrohemodynamic changes and many other signs, which are identical to those observed or usually detected in human septic patients, were present in this porcine model of intraabdominal sepsis. This has led us to conclude that this large animal model characterises the circulatory failure of human sepsis correctly, and may be used for further research of the disease and to test novel therapeutic opportunities.

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