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ORIGINAL ARTICLE

Pneumonia–induced sepsis in mice: temporal study of inflammatory and cardiovascular parameters

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SUMMARY

The aim of the present work is to provide a better comprehension of the pneumonia-induced sepsis model through temporal evaluation of several parameters, and thus identify the main factors that determine mortality in this model. *Klebsiella pneumoniae* was inoculated intratracheally in anesthetized Swiss male mice. Inflammatory and cardiovascular parameters were evaluated 6, 24 and 48 h after the insult. The results show that severity of infection and the mortality correlated with the amount of bacteria. Six, 24 and 48 h after inoculation, animals presented pathological changes in lungs, increase in cell number in the bronchoalveolar lavage, leukopenia, increase in TNF-α and IL-1β levels, hypotension and hyporesponsiveness to vasoconstrictors, the two latter characteristics of severe sepsis and septic shock. Significant numbers of bacteria in spleen and heart homogenates indicated infection spreading. Interestingly, NOS-2 expression appeared late after bacteria inoculation, whereas levels of NOS-1 and NOS-3 were unchanged. The high NOS-2 expression coincided with an exacerbated NO production in the infection focus and in plasma, as judging by nitrate + nitrite levels. This study shows that *K. pneumoniae* inoculation induces a systemic inflammatory response and cardiovascular alterations, which endures at least until 48 h. *K. pneumoniae*-induced lung infection is a clinically relevant animal model of sepsis and a better understanding of this model may help to increase the knowledge about sepsis pathophysiology.

Keywords
Klebsiella pneumoniae, mouse, nitric oxide, NOS-2, pneumonia, sepsis

Sepsis is the major cause of death in intensive care units, in spite of recent medical advances. It is considered a major health problem with a rising incidence and high mortality rate, besides consuming huge healthcare resources (Angus *et al.* 2001; Alberti *et al.* 2002). The development and progression of sepsis affects mainly the cardiovascular and immunological systems (Cohen 2002; Baron *et al.* 2006).

The study of sepsis and new therapeutic alternatives remains challenging due to the complexity of this syndrome. Animal models have been used to investigate the pathogenesis of sepsis and septic shock and have been tested for potential therapeutics. However, several therapies that worked in animals have failed in human sepsis trials (McCloskey *et al.* 1994; Bone *et al.* 1995; Fisher *et al.* 1996). Although the transposition of animal data to the clinical setting is intrinsically difficult, some of these failures may be attributable to the use of inadequate models.

Sepsis models can be broadly divided into three categories: exogenous administration of a toxin (such as zymosan or bacterial lipopolysaccharide), exogenous administration of a viable pathogen (bacteria is the most used) and breaches in the animal endogenous protective barriers (such as cecal ligation and puncture, CLP and colon ascendens stent peritonitis, CASP models; for an excellent review, see Buras *et al.* 2005). Whereas models such as CLP and CASP are helpful in understanding polymicrobial sepsis, human sepsis is more frequently caused by a single pathogen (Alberti *et al.* 2002).

Pneumonia is one of the major causes of sepsis, being responsible for almost 50% of all sources of infection

(Angus *et al.* 2001; Alberti *et al.* 2002). There are a number of studies concerning lung bacterial infection. However, studies are in general directed to the organ site (lungs) or pathogens with tropism for the lungs. Most of the reports in this field fall in one of three categories: (i) an infection restricted to the lungs (Soares *et al.* 2002); (ii) inoculation of bacteria that causes pneumonia into the peritoneal cavity (Greisman *et al.* 1979; Sutherland *et al.* 2008; Kumar & Chhibber 2011); and (iii) intravenous infusion of these same pathogens (Greisman *et al.* 1979; Wang *et al.* 2001; Cogen & Moore 2009). Other studies use a combination of burn injury and pulmonary sepsis (Davis *et al.* 2004; Horton *et al.* 2007) or pneumonia after CLP procedure (Muenzer *et al.* 2006), aimed to mimic nosocomial infections that result from immune depression. In addition, it is usual that only the lung infectious focus is studied, without systemic or temporal evaluations.

Therefore, taking into account that pneumonia-induced sepsis model has not been systematically studied and given the importance of a better understanding of sepsis models to increase the success of animal data transposition to clinical trials, the main goal of the present report is to study inflammatory and haemodynamic changes that occur over time in a pneumonia-induced sepsis in mice.

Material and methods

Animals

Male Swiss mice (weighing 35–40 g) were housed in a temperature- and light-controlled room (23 ± 2 °C; 12-h light/dark cycle), with free access to water and food.

Ethical Approval

All procedures used in this report have been approved by Universidade Federal de Santa Catarina Institutional Committee for Animal Use in Research and are in accordance to the Brazilian Government Guidelines for Animal Use in Research (CONCEA).

Bacterial inoculum

The bacterium used was *Klebsiella pneumoniae* - ATCC 700603 (American Type Culture Collection, Rockville, MD, USA). Bacteria were made pathogenic by 10 passages in C57/Bl6 mice (intratracheal injection and collection of the spleen 24 h later) and kept frozen in a −80 °C freezer in brain heart infusion (BHI) broth containing 10% glycerol until use. Bacteria were frozen in the log phase of growth. Before each experiment, individual aliquots were thawed, washed twice with sterile Dulbecco's phosphate-buffered saline (PBS, in mM 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 $NaHPO_4$; pH 7.4), suspended in BHI broth and incubated 18 h at 37 °C in the presence of 5% CO_2. The broth was then centrifuged and the resulting pellet was washed twice with sterile PBS. Bacterial concentration was determined by measuring the absorbance at 600 nm and comparing to a standard curve plotting optical density of bacteria suspension and colony-forming units (CFU) of *K. pneumoniae* plated on Mueller-Hinton agar. Bacteria were then diluted in sterile PBS to the desired concentration for inoculation.

Induction of pneumonia

Mice were anesthetized with tribromoethanol and ketamine (375/25 mg/kg i.p.) and placed in supine position. Under aseptic conditions, a 5 mm vertical incision was made in the skin of the neck. The trachea was identified and 0.05 ml of either sterile PBS (sham-inoculated) or bacterial suspension (pneumonia) was injected into the trachea with a sterile 30-gauge needle. Skin was sutured and animals were left for recovery in a warm cage. For survival experiments, the number of viable bacterial inoculated into the trachea was 1, 2, 3, 4, 5 and 10 x 10^8 CFU. Survival rate was recorded every 12 h over a 3-day period. Based on the results of this experiment, the dose of 4 × 10^8 CFU was chosen for the remainder of experiments because it induced ~50% death. All other experiments were performed 6, 24 and 48 h after bacterial inoculation. Animals received 30 ml/kg of sterile warm PBS subcutaneously. Until anaesthesia recovery, animals were maintained at 37 °C in their cages, and afterward were housed in a temperature- and light-controlled room (23 ± 1 °C; 12-h light/dark cycle), with free access to water and food.

Mean arterial pressure (MAP) measurement

Under anaesthesia with ketamine and xylazine (100 and 15 mg/kg, respectively, supplemented at 45- to 60-min intervals), heparinized PE-10 polyethylene catheters were inserted into the left femoral vein for drug injections and into the right carotid artery for recording of mean arterial pressure (MAP). To prevent clotting, a bolus dose of heparin (30 IU) was injected immediately after vein cannulation. Animals were allowed to breathe spontaneously via a tracheal cannula. Body temperature was monitored by a rectal thermometer and maintained at 37 ± 1 °C by means of a heating table. At the end of the experiment, animals were sacrificed with anaesthesia overdose. Blood pressure data were recorded with a catheter pressure transducer (Mikro-Tip®, Millar Instruments, Inc., Houston, TX, USA) coupled to a Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). Results were expressed as mean ± SEM of the peak changes in MAP (as mmHg) relative to baseline, and recorded following administration of a given compound.

Histology of lungs

Animals were killed 6, 24 and 48 h after surgery and lung tissues were harvested, fixed with neutral-buffered formalin for 24 h, and embedded in paraffin. Right lung lobe sections (5 μm) were cut and stained with haematoxylin-eosin (HE)

and examined under light microscopy (Model DMI 3000B, Leica Microsystems, Wetzlar, Germany). Lungs were scored on the basis of the degree and extent of inflammation, as previously described (Beck *et al.* 2001). The whole lung was evaluated using the Beck's score system, and the images chosen were representative of the mean score of the group. Three independent investigators, without knowledge of the experimental groups, graded semi-quantitatively the extent of inflammation according to the relative degree of inflammatory infiltration. The scoring is as follows: 0, no inflammation; 1, mild influx of inflammatory cells; 2, increased inflammation with approximately 25–50% of the total lung involved; 3, severe inflammation involving 50–75% of the lung; 4, almost all lung tissue contains inflammatory infiltrate.

BAL and blood cell counts

Blood was obtained from animals through cardiac puncture. To recover intrapulmonary inflammatory cells, 1 ml of sterile PBS was instilled into the lungs through the trachea, and bronchoalveolar lavage fluid (BAL) was aspirated back. This procedure was repeated three times. Only the samples in which recovery was at least 80% of the injected volume were analysed. Blood and BAL cell numbers were determined with a haemocytometer, and differential counts were determined on Cytospin smears stained with rapid Panoptic (Laborclin, Pinhais, PR, Brazil). Blood and BAL were centrifuged at 1200 ***g*** for 10 min at 4 °C, and the supernatants were saved for further cytokine analysis.

Quantification of bacterial load

Hearts and spleens were aseptically harvested, placed in 100 μl of sterile PBS and homogenized. Blood and BAL were also obtained under sterile conditions and were diluted serially (1:10) in sterile PBS. Ten microlitres of each dilution or tissue homogenate was aseptically plated and cultured on Mueller-Hinton agar dishes at 37 °C. After 24 h, the number of bacterial colonies was counted.

Determination of cytokine levels

Blood and BAL were assayed for TNF-α and IL-1β levels using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's recommendations (PreproTech Inc, Rocky Hill, NJ, USA).

Creatinine, lactate, AST and ALT levels

Plasma levels of creatinine, lactate, AST and ALT were measured using commercially available clinical assay kits (Bio-Clin, Belo Horizonte, MG, Brazil).

NOx (nitrite + nitrate) assay

Briefly, zinc sulphate-deproteinized plasma samples were subjected to nitrate conversion. Nitrate was converted to nitrite using *E. coli* nitrate reductase for 3 h at 37 °C. Samples were centrifuged for bacteria removal, and 100 μl of each sample was mixed with Griess reagent (1% sulphanilamide in 10% phosphoric acid/0.1% naphtylethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm in a plate reader. Standard curves of nitrite and nitrate (0–150 μM) were run simultaneously. Values are expressed as μM NOx (nitrate + nitrite).

Western blotting of nitric oxide synthases (NOS-1, NOS-2 and NOS-3)

Lung and heart tissues were quickly frozen in liquid nitrogen, homogenized in a lysis buffer (40 mM HEPES, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride), centrifuged at 10,000 ***g*** and the supernatant collected. Protein samples (50 μg/lane) were subjected to denaturing gel electrophoresis (SDS/PAGE, 7% gel). After electrophoresis, proteins were electro-transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with T-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.8 mM $Na_2HPO_4.2H_2O$ and 0.05% Tween-20, pH 7.4) containing 5% skimmed milk followed by incubation with rabbit polyclonal anti-NOS-1, NOS-2, NOS-3 or actin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Following washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Amersham Biosciences, Piscataway, NJ, USA) for 1 h at room temperature. Immune complexes were visualized by chemiluminescence, and band intensity was quantified by densitometry using ImageJ® software (available as public domain from US National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean ± SEM of n animals (*n* value is indicated in Figure legends). Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by the appropriate post hoc test, as indicated in the Figure legends. Differences in the survival were determined with log rank test, and lung pathology score was analysed by Kruskal–Wallis test. A *P* value of less than 0.05 was considered significant. Statistical tests were performed using GRAPHPAD PRISM® software (San Diego, CA, USA).

Results

Mortality and cardiovascular parameters following Klebsiella pneumoniae *inoculation*

We investigated the relationship between colony-forming units (CFU) of *K. pneumoniae* and mortality rate. As shown in Figure 1, the death toll correlated almost linearly with the number of injected bacterial CFU. Survival rate of animals were 100%, 85%, 57%, 42%, 15% and none (all animals died until 60 h) for 1, 2, 3, 4, 5 and 10×10^8 CFU/

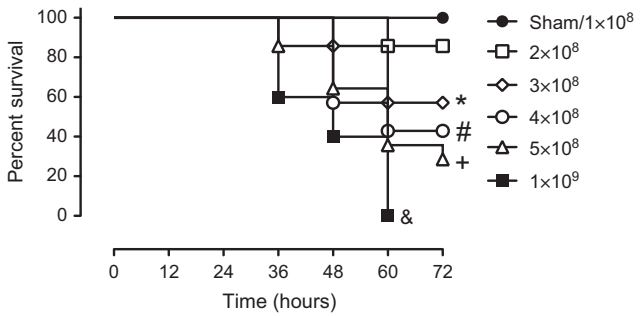

Figure 1 Survival following *Klebsiella pneumoniae* inoculation in mice. Mice were inoculated intratracheally (i.t.) with increasing amounts of bacterial CFU; sham-inoculated animals received PBS and exhibited no mortality. Statistical analysis was performed using log rank test. * $P = 0.046$; # $P = 0.0159$; + $P = 0.0034$; and & $P = 0.0004$ compared with sham group, $n = 8$ per group. Results were expressed as per cent survival and are representative of two different experiments.

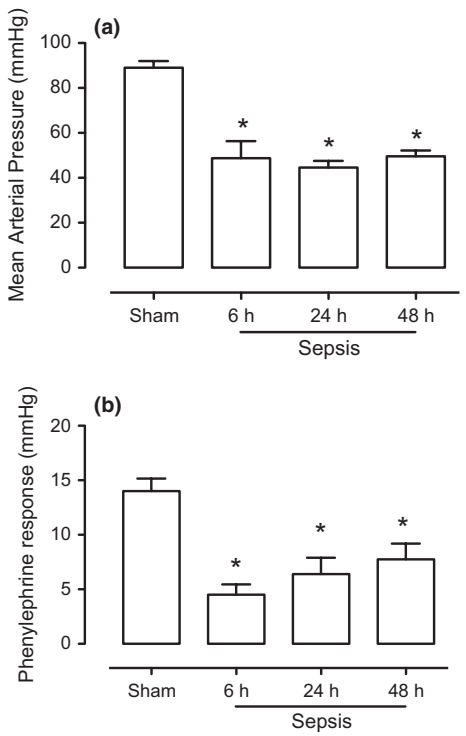

Figure 2 Vascular dysfunction in mice inoculated with *K. pneumoniae*. Mice received 4×10^8 CFU of *Klebsiella pneumoniae* i.t. and six, 24 and 48 h after inoculation, mean arterial pressure (Panel A) and phenylephrine response (10 nmol/kg; i.v.; Panel B) were evaluated. Sham-inoculated animals were evaluated at 6, 24 and 48 h and because there was no difference among them, they are presented as one group. Each bar represents the mean ± SEM of 6–8 animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ compared to sham group.

animal, respectively. All sham-inoculated animals survived. For the following experiments, the concentration of 4×10^8 CFU/animal was chosen, and the parameters were studied 6, 24 and 48 h after bacterial inoculation.

The main cardiovascular characteristics of sepsis and septic shock are hypotension and hyporesponsiveness to vasoconstrictors. We observed that 6 h, 24 h and 48 h after surgery, animals inoculated with *K. pneumoniae* developed a sustained hypotension (Figure 2, Panel A) and hyporesponsiveness to the vasoconstrictor phenylephrine when compared to sham-inoculated animals (Figure 2, Panel B).

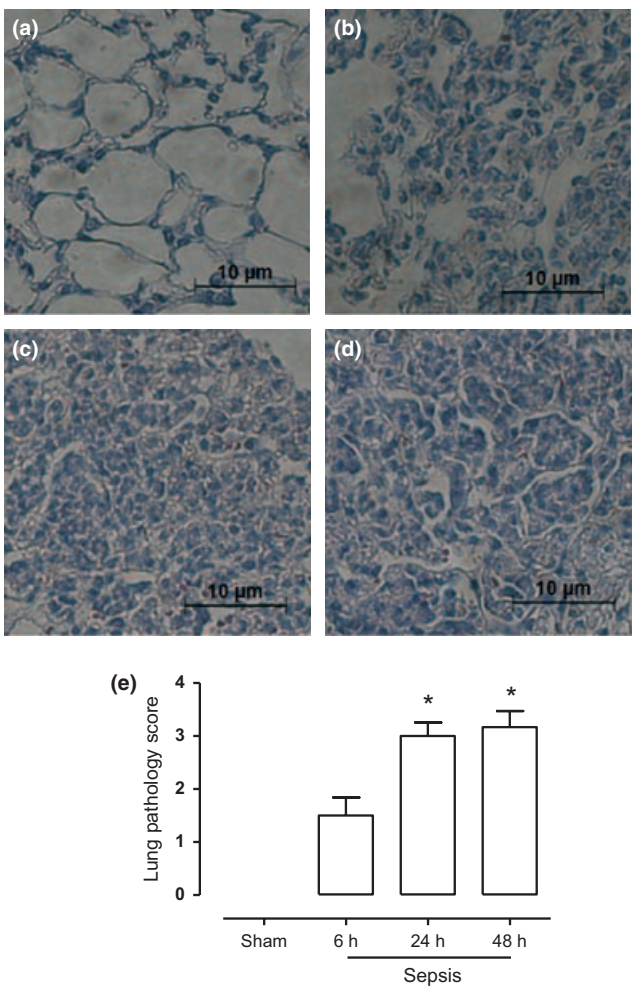

Figure 3 Lung histology and pathology score. Mice received 4×10^8 CFU of *K. pneumoniae*. Six (Panel B), 24 (Panel C) and 48 h (Panel D) after inoculation animals were terminally anaesthetized and lungs were harvested. Lung sections were obtained, stained with HE and examined under light microscopy (Leica DMI 3000B). A representative section of the lung of sham-inoculated animals is shown in Panel A. The pathology score is shown in Panel E and was obtained as detailed in Methods section. Each bar represents the mean ± SEM of six animals. Statistical analysis was performed using Kruskal–Wallis followed by Dunn's post hoc test. * $P < 0.05$ when compared to sham animals.

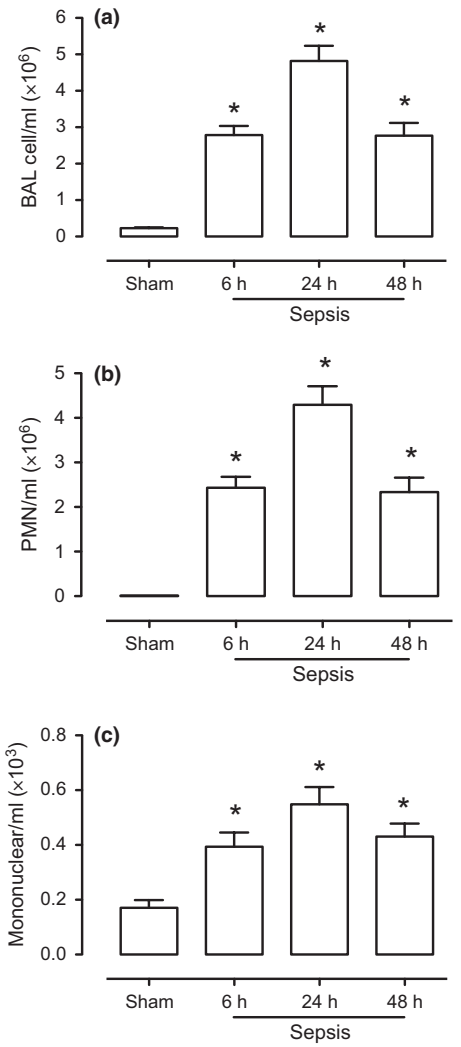

Figure 4 Cell composition of brochoalveolar lavage of mice inoculated with *K. pneumoniae*. Six, 24 and 48 h after i.t. inoculation of 4×10^8 CFU or PBS, mice were terminally anaesthetized, and BAL was obtained as detailed in Methods section. BAL cell count is shown in Panel A, polymorphonuclear neutrophil counts in Panel B and mononuclear cell counts in Panel C. Data are expressed as mean ± SEM of seven animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ when compared to sham animals.

Lung pathology and cellular composition of bronchoalveolar lavage

To evaluate the impact of the infection histology of lung tissue and the cellular composition of BAL were studied. Figure 3 depicts representative pictures of lungs sections of mice inoculated with bacteria when examined 6 (Panel B), 24 (Panel C) and 48 (Panel D) hours after inoculation. The lung histology of a control (trachea inoculated with saline) animal is shown in Panel A. Six hours after bacterial inoculation, lungs presented inflammatory characteristics, but still with a lower magnitude. A large degree of cellular infiltration and inflammatory exudation was seen in the lungs 24 and 48 h after infection. In addition, alveoli were grossly distorted, filled with inflammatory cells and the gas exchange membrane was thickened (Figure 3). The quantification of the lung damage is shown in Figure 3, Panel E.

To evaluate which was the main inflammatory cell in BAL of septic animals, we performed total and differential counts in the samples. Inflammatory cell content was observed in BAL as early as 6 h, peaked at 24 h and start decreasing 48 h after bacteria inoculation (Figure 4, Panel A). The majority of infiltrating cells were polymorphonuclear leucocytes (Figure 4, Panel B). Albeit in lesser numbers, mononuclear cells displayed the same time course (Figure 4, Panel C). When blood leucocytes were examined, there was an initial leukopenia (6 and 24 h after inoculation), and by 48 h, the numbers were similar to control animals (Figure 5, Panel A). This leukopenia was caused by the reduction in polymorphonuclear and monocyte numbers (Figure 5, Panels B and D), whereas lymphocyte numbers remained relatively constant (Figure 5, Panel C).

Infection, inflammation and organ damage

We evaluated the infectious process and the spread of bacteria in septic animals. In Figure 6, it is shown the bacterial load in BAL (Panel A), blood (Panel B) and homogenates of spleen (Panel C) and heart (Panel D). As can be observed, although the infection was rapidly cleared in the blood, it persisted for at least 48 h in BAL and organs. The presence of systemic inflammatory cytokines is an important marker of sepsis. The two more conspicuous markers of systemic inflammation are TNF-α and IL-1β. Both cytokines were increased in BAL and plasma of septic animals (Figure 7). TNF-α levels rose very early (6 h) and declined acutely in BAL, whereas its peak in plasma was 24 h after inoculation. IL-1β also increased early after infection, but its levels remained high in both BAL and plasma until the end of the experimental period. We also studied organ damage markers in this model. Tissue hypoperfusion peaked 24 h and remained so 48 h after bacterial inoculation, as judged by plasma lactate levels (Figure 8, Panel A). *K. pneumoniae* infection induced liver damage as evidenced by increases in plasma levels of aspartate transaminase (AST; Figure 8, Panel C) and alanine transaminase (ALT; Figure 8, Panel D). Renal damage was evidenced by higher creatinine levels 24 h after inoculation (Figure 8, Panel B). Another important marker of sepsis is NO production by NO synthases, mainly NOS-2. For this, NOx (nitrite + nitrate) levels in BAL and plasma of septic animals were measured, as well as the profile of NOS expression in the infection focus and heart tissue. BAL and plasma NOx levels started to increase 24 h after sepsis induction with a steep increase 48 h after bacteria inoculation (Figure 9). When NO synthases expression was evaluated in lung

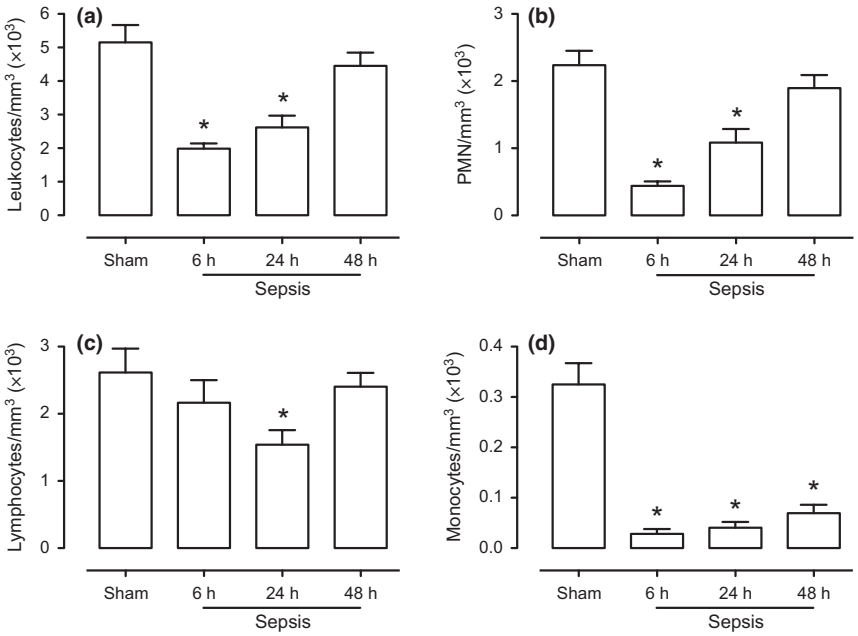

Figure 5 Blood leukocyte composition of mice inoculated with *K. pneumoniae*. Six, 24 and 48 h after i.t. inoculation of 4×10^8 CFU or PBS, mice were terminally anaesthetized, and blood was obtained by cardiac puncture. Total cell number is shown in Panel A, polymorphonuclear neutrophil counts in Panel B, lymphocyte counts in Panel C and mononuclear cell counts in Panel D. Data represent the mean ± SEM of seven animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ when compared to sham animals.

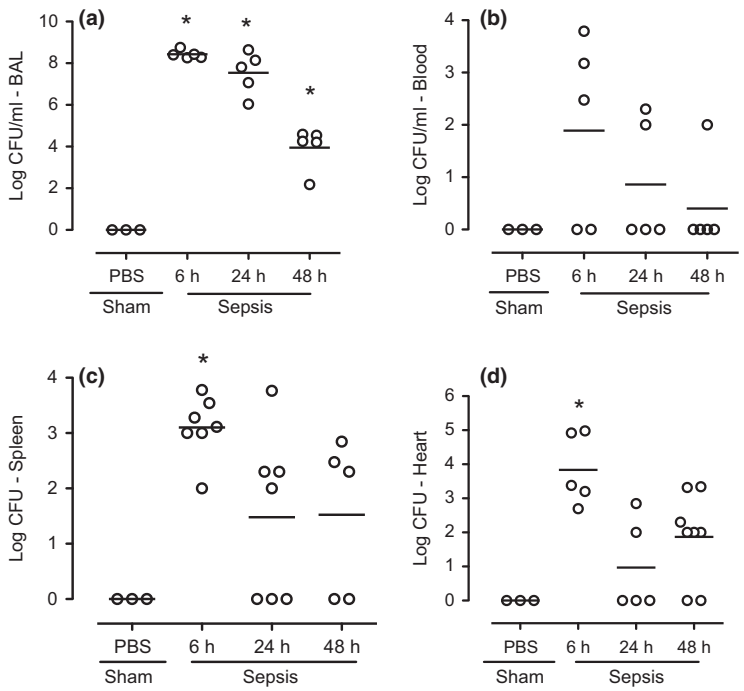

Figure 6 Bacterial load in BAL, blood, spleen and heart of mice inoculated with *K. pneumoniae*. Mice received 4×10^8 CFU of *K. pneumoniae* i.t. and 6, 24 and 48 h after inoculation, BAL (Panel A), blood (Panel B), spleen (Panel C) and heart (Panel D) were collected, tissues were homogenized and serial dilutions were placed on Mueller-Hinton agar plates. Colony-forming units (CFU) were determined 24 h after plating and were expressed as log CFU. Individual animals are represented by individual symbols, and the horizontal bar is the mean. Sham-inoculated animals received PBS. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ compared to sham group.

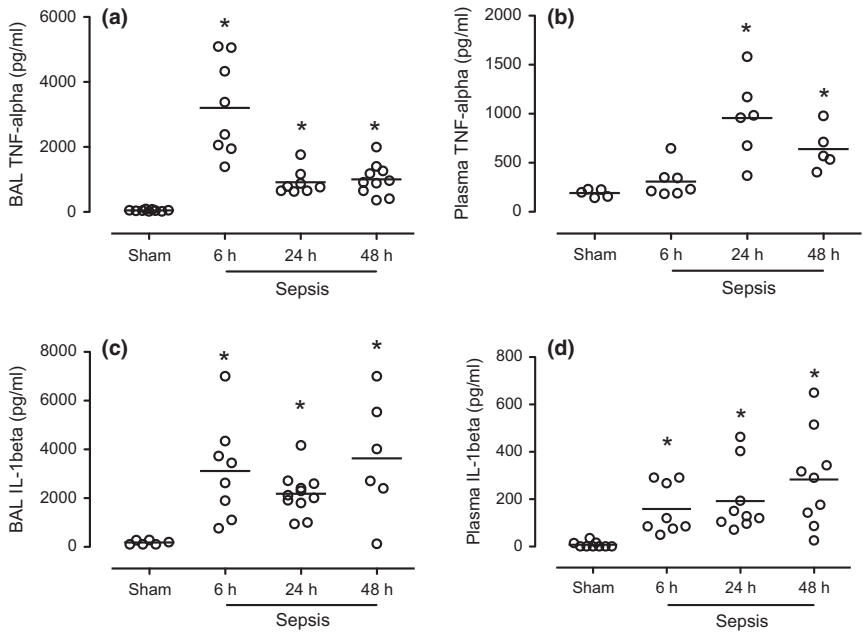

Figure 7 TNF-alpha and IL-1beta levels in BAL and plasma of mice inoculated with *K. pneumoniae*. Mice received 4×10^8 CFU of *K. pneumoniae* and 6, 24 and 48 h after surgery, BAL and plasma were assayed for TNF-alpha (Panels A and B) and IL-1 beta (Panels C and D). Each symbol represents one individual animal, and the horizontal bar is the mean. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ compared to sham group.

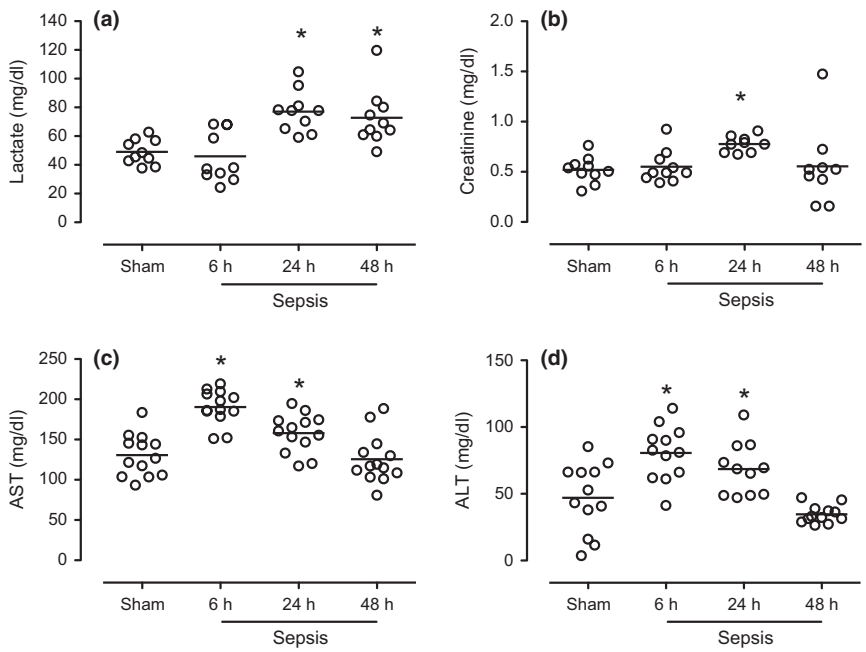

Figure 8 Organ damage markers of mice inoculated with *K. pneumoniae*. Mice received 4×10^8 CFU of *K. pneumoniae*, and at the shown periods after inoculation, plasma samples were collected and assayed for tissue damage markers. Each symbol represents one individual animal, and the horizontal bar is the mean. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ when compared to sham animals.

(Figure 10, Panel A) and heart tissues (Figure 10, Panel B), an increase NOS-2 expression was observed 48 h after the infection, mainly in lungs (Figure 10, Panel E). Although NOS-2 in heart tissue also increased (Figure 10, Panel F), this increase was not statistically significant. NOS-1 (Figure 10, Panels C and D) and NOS-3

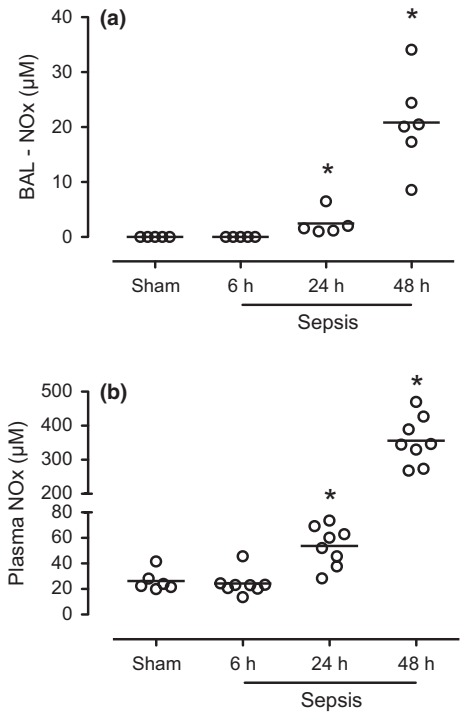

Figure 9 Nitrite + nitrate (NOx) levels in BAL and plasma of mice inoculated with *K. pneumoniae*. Mice received 4×10^8 CFU of *K. pneumoniae* and BAL and plasma samples were collected and assayed for NOx levels. Each symbol represents one individual animal, and the horizontal bar is the mean. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ when compared to sham animals.

(Figure 10, Panels G and H) expression in lungs and heart tissue were not different between the groups.

Discussion

In the present report, we describe a time course study of a mouse model of sepsis induced by gram-negative pneumonia. Our interest in studying this model is twofold: first, the reports showing that pneumosepsis models are generally characterized on the basis of lung pathology and not on the systemic host response, and second, reports in general study only one time point after infection, and our interest was in to study how changes happen over time.

Lungs are the most common sites of infection in sepsis, accounting for almost 50% of all sources of infection (Angus *et al.* 2001; Alberti *et al.* 2002). Moreover, the main microorganisms responsible for gram-negative sepsis are *Enterobacteriaceae* (such as *Escherichia coli* and *Klebsiella* species) and *K. pneumoniae* infections and are mainly responsible for nosocomial pneumonia (Alberti *et al.* 2002; Tsiotou *et al.* 2005).

Interestingly, in this model, we did not observe any mortality up to 36 h after bacteria inoculation. This slow-developing mortality may be an advantage in comparison with other sepsis models that exhibit a significant mortality within the first 6 to 12 h after injury and hence may not reproduce the time course and outcome typical of human sepsis (Piper *et al.* 1996). Another advantage of the present model is that the mortality correlates very well with the number of CFU injected. Mice that received 1×10^8 CFU into lungs exhibited 100% survival, those who received 4×10^8 CFU exhibited a little more than 50% of mortality, while animals that received 1×10^9 CFU, exhibited 100% mortality by 60 h. In other models of sepsis, such as CLP, for example, the reproducibility is more difficult to be attained because the degree of cecal ligation and the amount of intestinal content that leaks from the punctures may vary between different laboratories, and even among researchers from the same laboratory (Buras *et al.* 2005). We have chosen to perform the subsequent experiments with 4×10^8 CFU, which induced ~50% mortality with an excellent reproducibility. With this amount of bacteria, animals clearly presented visual signs of sepsis such as piloerection, lethargy and tachypnea. Finally and distinct from other reports, we opted to monitor the levels of several parameters 6, 24 and 48 h instead of 'taking a picture' at only one time point. This temporal evaluation allowed us to have an insight into the evolution of sepsis over time.

Hypotension and hyporesponsiveness to vasoconstrictors are markers of vascular dysfunction in sepsis and septic shock (O'Brien *et al.* 2007; Fernandes & Assreuy 2008). When compared to control animals, mice inoculated with *K. pneumoniae* developed hypotension and a substantial reduction in the response to phenylephrine. Interestingly, the vascular dysfunction observed in this model is more intense and long lasting than that seen in CLP (Fernandes *et al.* 2009; Sordi *et al.* 2010, 2011). As in the CLP, the vascular dysfunction may be contributing to organ dysfunction, confirmed by markers of tissue hypoxia and liver/kidney damage found in the present report.

Concerning the inflammatory cell component of the present model, histological analysis has shown that neutrophils adhere to the injured capillary endothelium and transmigrate through the interstitium into the air space. The invasion of lungs by neutrophils started as early as 6 h after sepsis induction and peaked 24 h after inoculation. We observed a large degree of cellular infiltration and inflammatory exudate in all time points studied. The time of neutrophil migration to the infectious focus coincided with the development of a substantial leukopenia in animals, suggesting that both events may be related.

As expected, the number of bacteria found in BAL was high in all the studied periods. More important, however, the infection was not restricted to the inoculation site, because we have found bacteraemia and colony-forming units in spleen and heart tissues. This finding is perhaps the strongest evidence that this model is indeed producing systemic infection and inflammation. Although the presence of bacteria in the bloodstream was transient, detectable bacteraemia does not seem to be essential for the development of septic shock. For instance, only 30–50% of patients with sepsis present positive blood culture and in about 30% of

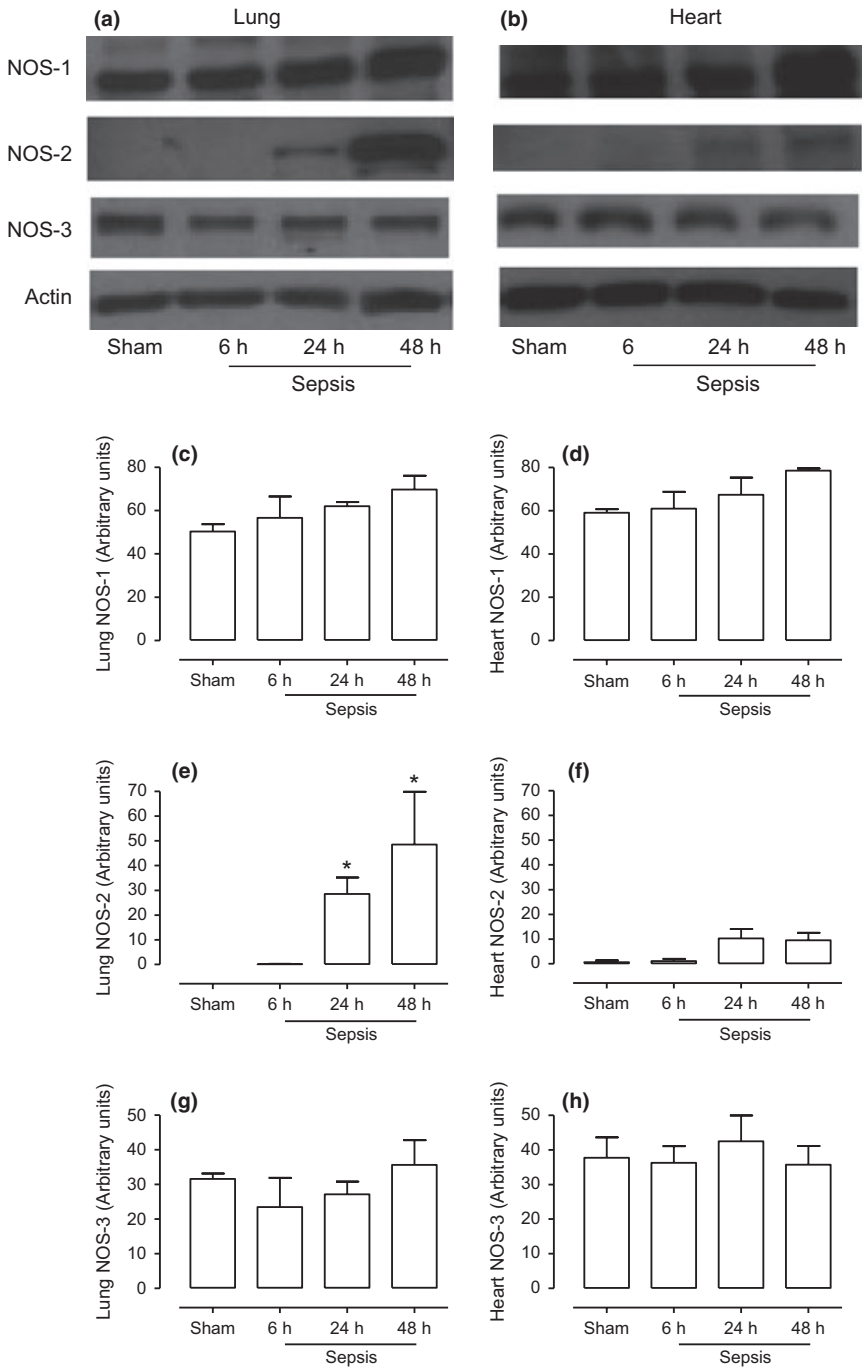

Figure 10 Western blotting of NOS-1, NOS-2 and NOS-3 in lung and heart tissues of mice inoculated with *K. pneumoniae*. Tissues were harvested and homogenized, and the same amount of protein (50 μg) was loaded in each well. Representative immunoelectrophoresis of lung (Panel A) and heart (Panel B) of NOS-1, NOS-2, NOS-3 and actin is shown. Band intensity of NOS-1 (Panels C and D), NOS-2 (Panels E and F) and NOS-3 (Panels G and H) of lung (left) and heart tissues (right) were quantified by densitometry. Each bar represents the mean of three independent experiments of 4 animals and vertical lines are SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. * $P < 0.05$ when compared to sham animals.

patients, the site of infection is not determined (Kieft *et al.* 1993). The quick clearance of bacteria from the bloodstream and its preference for certain anatomical location is a well-described event (Wicher *et al.* 1996; Al-Banna *et al.* 2008), and it is possible that *K. pneumoniae* is only transiently present in the bloodstream en route to tissues such as spleen and heart. The presence of an infectious focus is more clinically relevant and represents an advantage of this model. Endotoxemia and injection of bacteria directly in the bloodstream are models without an infectious focus,

and although more controlled and standardized, they do not reproduce the real characteristics of human sepsis (reviewed in Poli-de-Figueiredo *et al.* 2008). Therefore, different from endotoxin administration where the significance as a model of sepsis is controversial, being more an endotoxicosis model, inoculation of *K. pneumoniae* provides a spreading infection from a defined infection focus.

Cytokines are key elements in endotoxemia, sepsis and septic shock, being a very common feature in systemic inflammatory response (Eskandari *et al.* 1992; Walley *et al.* 1996; Tsiotou *et al.* 2005). Endotoxemia by lipopolysaccharide injection is characterized by rapid and transient, albeit very high, plasma cytokine levels (Deitch 1998). The model described here induced a long-lasting increase in systemic inflammatory cytokines levels. In this regard, the present model seems to reproduce more closely the sustained inflammatory response of human sepsis.

An important mediator of sepsis is NO, which is involved in almost all host responses to infection such as inflammation, vascular dysfunction, increased vascular permeability, migration and activation of leucocytes and microorganism killing (reviewed in Assreuy 2006; Fernandes & Assreuy 2008). Twenty-four hours after inoculation, levels of NO metabolites (NOx) in plasma and BAL levels were high, but this increase is far more significant at later stages of sepsis. The excessive production of NO may exert cytotoxic effects through peroxynitrite (ONOO-) production, which in turn may cause cell damage (Szabó 1996; Lange *et al.* 2010). Although NO is an important tool for infection control by the host (Moncada *et al.* 1991), it can cause a severe deterioration of the pulmonary gas exchange, leading animals to death. The high increase of NO metabolites coincided with the expression of NOS-2, greatly enhanced 48 h after infection.

Although we have observed NOS-2 expression in lung tissue, we did not investigate its cellular source. NOS-2 expression in endotoxemia models depends on neutrophil sequestration within the lung vasculature (Gebska *et al.* 2005). On the other hand, previous works have demonstrated that NOS-2 expression in lung tissue occurs mainly in epithelial cells (Hjoberg *et al.* 2004), especially type II cell (Sunil *et al.* 2002). We also performed myeloperoxidase (MPO) assay in lung tissue and, similar to neutrophil profile in BAL cells, MPO activity remained elevated in all time points (data not shown) thus not coinciding with NOS-2 expression profile. In this way, we believe that epithelial cells are the main source of NOS-2 and NO in this model.

Nitric oxide and NOS-2 increase has already been reported in other sepsis models (Preiser *et al.* 2001; Kan *et al.* 2004), and in both studies, NOS-2 expression occurred early, within few hours. Lange and colleagues reported a time course of NO synthases in an ovine sepsis model, but they did not correlate NO production with mortality, and their observations were made only until 24 h after injury (Lange *et al.* 2010). The results presented here show that in pneumonia-induced sepsis, NOS-2 expression and mortality are protracted and develop slowly, compared to the CLP model where NOS-2 is found few hours after surgery (Wu *et al.* 2003; R Sordi and J Assreuy, unpublished work). Therefore, our results suggest that the late NOS-2 expression, together with other inflammatory mediators, may also contribute to the mortality in this sepsis model. Distinct from NOS-2, NOS-1 and NOS-3 isoforms were not substantially altered in pneumonia-induced sepsis.

The death of animals was probably caused by the sum of several factors. First, the infectious process is an important aspect because the mortality correlates linearly with the number of bacteria injected, a similar finding in the clinical setting where patient outcome is dependent on infection intensity (Alberti *et al.* 2002). Second, as patients frequently die sterile (Alberti *et al.* 2002; Baron *et al.* 2006), the uncontrolled inflammatory response may have a crucial role in the mortality. It is well known that sepsis cause a substantial release of damage-associated molecular patterns (DAMPs), which leads to tissue and cell damage and trigger similar responses to pathogens in sepsis (Bianchi 2007; Zhang *et al.* 2010).

The present model has limitations that should be taken into account. First, there are many important differences between mice and human lung infection, ranging from the anatomy to the immunology (for a review, see Mizgerd & Skerrett 2008). A second limitation concerns the mouse strain, because some strains such as C57/Bl6 are far more susceptible to *Klebsiella* infection (Schurr *et al.* 2005). Further studies comparing outbred (as ours) and inbred strains may be of importance in this regard. Finally, intra-tracheal infection methods do not provide a homogenous spread of the infection in the lung, because microorganisms tend to infect the base of lungs (Brain *et al.* 1976; Mizgerd & Skerrett 2008).

An interesting perception derived from working with different models of sepsis is that sepsis is multifactorial: several different causes can be responsible for its development, and therefore, different outcomes are expected depending on the origin of the process. For example, human sepsis initiated by pneumonia probably has a different outcome and requires distinct clinical measures compared with sepsis triggered by appendicitis (Deitch 1998). Indeed, the physiopathology of pneumosepsis is distinct from an abdominal sepsis (Deitch 1998; Buras *et al.* 2005). Clinical trials usually do not consider this and group septic patients together independently of the source of the infection and the responsible agent. Maybe this fact also contributes to the failure of several therapies that work in animals but fail in human sepsis.

In summary, inoculation of *K. pneumoniae* seems an interesting model for studying both inflammatory and cardiovascular alterations that occurs in sepsis and may help to understand this complex syndrome, aiming to improve the efficacy of clinical therapies.

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Conflict of Interest

The authors declare no conflict of interest.

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