Protective effect of procysteine on Acinetobacter pneumonia in hyperoxic conditions

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Objectives: Ventilator-associated pneumonia (VAP) is an important cause of morbidity and mortality in critical care settings. Acinetobacter has become a leading cause of VAP. In particular, the appearance and spread of multidrug-resistant Acinetobacter is of great concern. In this study, we examined the effect of the antioxidant procysteine on Acinetobacter murine pneumonia in hyperoxic conditions in order to simulate VAP.

Methods: Acinetobacter was administered intranasally to BALB/c mice kept in hyperoxic conditions. At designated timepoints, bacterial number, cytokine production and histopathological findings in the lungs were examined. The effects of procysteine on survival rates, lung bacterial burdens and the phagocytic activities of alveolar macrophages were evaluated.

Results: Drastic decreases in survival were observed when the infected mice were kept in hyperoxic conditions (P < 0.001). Significant differences in pulmonary bacterial number and neutrophil accumulation were observed between mice kept in hyperoxic or normoxic conditions on day 3. Although all mice infected with Acinetobacter spp. and kept in hyperoxic conditions died by day 3, procysteine treatment significantly improved survival (60% survival on day 7, P < 0.01). Procysteine treatment decreased the lung bacterial burden on days 2 and 3. Finally, improved uptake of FITC-labelled beads by alveolar macrophages from mice treated with procysteine and kept in hyperoxic conditions was noted.

Conclusions: These results suggest that hyperoxia increases mortality in mice with Acinetobacter pneumonia and that procysteine improves survival by increasing the phagocytic activity of alveolar macrophages in mice kept in hyperoxic conditions.

Keywords: phagocytic activity, alveolar macrophages, ventilator-associated pneumonia, VAP

Introduction

Ventilator-associated pneumonia (VAP) remains a leading cause of nosocomial infections in intensive care units, despite management care and the development of antimicrobial therapy for pneumonia. It has been reported that VAP occurs in 9%–27% of all intubated patients and has one of the highest mortality rates (25%–50%).1–4 It is considered that the pathogenesis of VAP involves the colonization of the aerodigestive tract with pathogenic bacteria and the aspiration of contaminated secretions.5 Most cases of VAP are caused by common pathogens, such as Pseudomonas spp., Acinetobacter spp., staphylococci, Enterobacteriaceae and streptococci.

In addition, VAP is a common complication of acute respiratory distress syndrome (ARDS) or acute lung injury (ALI), which are treated by administering a high concentration of oxygen. Although oxygen supplementation is useful for VAP patients with severe hypoxaemia, prolonged or even transient administration of oxygen may promote cellular damage and tissue injury. Our previous studies demonstrated that hyperoxia serves as an important...
cofactor for the development of ALI and lethality in cases of Legionella pneumophilia infection and for bacterial dissemination of Pseudomonas aeruginosa infection.\(^6,7\) VAP is likely to result from highly antibiotic-resistant organisms, such as P. aeruginosa and Acinetobacter spp., particularly in patients previously treated with antibiotics. Furthermore, Acinetobacter in VAP patients has emerged as one of the most problematic pathogens, because only a limited number of antibiotics are suitable for treatment targeting this organism.\(^8\)–\(^{11}\)

Oxidative stress induced by reactive oxygen species (ROS), which are chemically reactive molecules containing oxygen, plays a central role in the pathogenesis of several pulmonary diseases, including ARDS and ALI. Antioxidants are substances that may protect cells against the effects of free radicals, such as ROS. However, little is known about the effects of antioxidant supplements on VAP.

In the present study, we examined the effects of hyperoxia on lethality in mice with Acinetobacter pneumonia. Moreover, we assessed the protective roles of procysteine as an antioxidant on the pathogenesis of Acinetobacter pneumonia in mice kept in hyperoxic conditions.

**Methods**

**Animals**

Specific-pathogen-free 5- to 7-week-old female BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). All mice were housed in pathogen-free conditions within the animal care facility at Toho University School of Medicine until the day of sacrifice. Experiments were conducted according to our institution’s ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments. Animal protocols were approved by the institutional animal care and use committee (approval number: #10-52-54).

**Acinetobacter inoculation**

The Acinetobacter sp. used in this study was isolated from a clinical specimen at Toho University Omori Medical Center. The strain was identified as Acinetobacter sp. (GenBank GU566324) using 16S ribosomal DNA sequencing and a BLAST search. These bacteria were inoculated in Luria–Bertani broth and incubated for 20 h at 37\(^\circ\)C. The study animals were administered with one of two bacterial dosages: a sublethal (\(1.0\times10^8\) cfu/mouse) or lethal (\(1.0\times10^9\) cfu/mouse) dose. After intranasal administration of bacteria, mice were inoculated intraperitoneally with saline or 4 mg (160 mg/kg) of procysteine (\(4\)-carboxylic acid, Wako, Japan). The dose of procysteine in this study was determined by reference to a publication by Lee et al.\(^{12}\) Additional doses of procysteine or saline were administered on days 1, 2 and 3. Mice were kept in hyperoxic conditions for 72 h in an airtight chamber.

**Exposure of mice to oxygen**

After recovering from the effects of anesthesia, one group of mice was kept in hyperoxic conditions for 72 h in an airtight chamber, whereas another group was placed in a ventilated room. For hyperoxic exposure, the oxygen concentration in the chamber was maintained at 90% by regulating the flow of oxygen, which was monitored using an in-line oxygen analyzer (model D2, Beckman, Fullerton, CA, USA).

**Harvesting of lungs for analysis**

Lung homogenates were prepared according to the method of Tateda et al.\(^6\) Supernatants were collected and stored at \(-40\,\text{°C}\) until use.

**Histological evaluation**

After CO\(_2\) asphyxia, the lungs were inflated and fixed with 10% buffered formalin. Samples were embedded in paraffin, stained with haematoxylin and eosin (HE) and examined under a light microscope.

**Cytokine assay**

The levels of proinflammatory cytokines (interleukin-6 (IL-6) and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\))) in the lung homogenates were determined using Duo Set ELISA Kits (R&D Systems, Minneapolis, MN, USA).

**Phagocytosis assay**

Mice were kept in hyperoxic conditions for 72 h in an airtight chamber and then sacrificed. The trachea was exposed and intubated using a 1.0 mm outer diameter polyethylene catheter. Branchioalveolar lavage (BAL) was performed by instilling normal saline. Approximately 2 mL of BAL fluid (BALF) was retrieved per mouse and centrifuged at 1000 rpm for 10 min. The cell pellets were resuspended in RPMI 1640 medium and divided into Lab-Tek\(^{16}\) Chamber Slides (Nunc, Denmark). After 3 h, non-adherent cells were removed by changing the medium. Adherent cells were incubated with FITC-labelled latex minibeads (Polysciences, Warrington, PA, USA) (1:100) at 37\(^\circ\)C for 60 min. After incubation, the slides were analysed using a fluorescence microscope (BZ-9000 Keyence, Japan). The uptake of FITC-labelled latex minibeads was confirmed by z-series analysis. Uptake of the minibeads was determined by counting 50 macrophages/slide for each sample. Each group (a total of 150 macrophages) was determined by counting. A Cytospin 3 (Thermo Scientific) was used for the preparation of BAL cells for cell differentiation.

**Statistical analysis**

Statistical significance was determined using the unpaired, two-tailed \(t\)-test. Survival curves were constructed by the Kaplan–Meier method and analysed by log-rank tests. For all tests, differences were considered to be statistically significant when \(P\) values were <0.05.

**Results**

**Effect of hyperoxia on lethality and lung bacterial burden in Acinetobacter-infected mice**

Drastic decreases in survival were observed in the group of mice exposed to the hyperoxic environment, whereas no mortality was observed in mice infected with Acinetobacter sp. in normoxic conditions (\(P<0.001\)) (Figure 1a). Furthermore, no mortality was observed in mice exposed to hyperoxia alone in the absence of Acinetobacter sp. infection (data not shown). To determine the cause of increased lethality under hyperoxic conditions, lungs were harvested from mice euthanized on days 1, 2 and 3 after challenge with a sublethal dose of Acinetobacter sp. (3.6×10\(^7\) cfu/mouse) (Figure 1b). Mice kept in hyperoxic conditions exhibited a significant delay in the clearance of Acinetobacter sp. from the lungs compared with mice kept in normoxic conditions on day 3.
However, there was no statistical difference between bacterial numbers in mice kept in hyperoxic or normoxic conditions on days 1 and 2 (6.5 ± 0.2 versus 6.8 ± 0.1 log cfu/lung on day 1; 5.1 ± 0.2 versus 5.2 ± 0.2 log cfu/lung on day 2). Histologic examination of the lungs at 72 h post-inoculation revealed severe inflammation,
including neutrophil infiltration, in the lungs of mice kept in hyperoxic conditions (Figure 1c, upper left), whereas no significant inflammation was observed in the lungs of mice infected with Acinetobacter sp. kept in normoxic conditions (Figure 1c, upper right).

**Effect of procysteine on survival and lung bacterial burden in mice kept in hyperoxic conditions**

Figure 2(a) shows that mice kept in hyperoxic conditions started to die 48 h after inoculation of a lethal dose of Acinetobacter sp. and all mice died by 72 h. In contrast, administration of procysteine during inoculation with Acinetobacter sp. significantly improved the survival of mice (P<0.01). Next, we examined the bacterial number in the lungs of mice treated with procysteine on days 0, 1, 2 and 3 after challenge with Acinetobacter sp. (Figure 2b). The bacterial burden in the lungs of Acinetobacter-infected mice treated with procysteine and kept in hyperoxic conditions was significantly reduced compared with mice treated with saline on day 2 (5.2 ± 0.1 versus 4.7 ± 0.1 log cfu/lung, P<0.01) and on day 3 (4.3 ± 0.2 versus 3.4 ± 0.1 log cfu/lung, P<0.05). The bacterial burden in the lungs infected with A. baumannii ATCC 49139 was also found to be reduced by procysteine treatment, compared with that in mice treated with saline on day 3 (5.7 ± 0.1 versus 5.3 ± 0.1 log cfu/lung, P<0.05).

**Procysteine promoted survival in Acinetobacter-infected mice kept in hyperoxic conditions in a dose- and duration-dependent manner**

The infected mice were treated with various doses of procysteine once daily for 4 days. In the high-dose procysteine treatment group (160 mg/kg), survival improved significantly compared with untreated mice, whereas low-dose procysteine treatment (40 or 80 mg/kg) did not improve survival in Acinetobacter-infected mice kept in hyperoxic conditions (data not shown). Next, we demonstrated the duration dependency of the efficacy of procysteine treatment. Procysteine administration once daily for 4 days at the same time as infection with Acinetobacter sp. significantly improved the survival of mice (P<0.05). However, no significant beneficial effect on the survival of mice treated with procysteine for 4 days was seen before 24 h on days 1 or 2 (data not shown). These results suggest that procysteine may be effective in mice infected with Acinetobacter and kept in hyperoxic conditions and further suggest that the timing of procysteine administration may be a critical factor.

**Effect of procysteine on the production of proinflammatory cytokines due to hyperoxia**

We investigated the protective effect of procysteine on inflammation in the lungs of mice kept in hyperoxic conditions. The

**Figure 2.** Protective role of procysteine in a VAP model of Acinetobacter pneumonia. BALB/c mice were intranasally inoculated with A. baumannii (8.7×10^7 cfu/mouse) under hyperoxic conditions. (a) Intraperitoneal administration of procysteine (160 mg/kg, n=11, filled triangles) or saline (n=11, filled circles) was performed from day 0 to day 3 and survival was then determined. **P<0.01 in comparison with the control group treated with saline. (b) The bacterial burden in the lung was determined on days 1, 2 and 3. All bars indicate mean ± SEM (n=7 in each group). **P<0.01 or *P<0.05 in comparison with the untreated group.
production of IL-6 and TNF-α was examined on days 2 and 3 (n = 5 in each group) after infection with A. baumannii (1.2 × 10⁶ cfu/mouse). No significant difference was observed in IL-6 (267.7 ± 66.5 versus 220.2 ± 54.4 pg/mL) and TNF-α (246.9 ± 28.6 versus 210.4 ± 23.9 pg/mL) production in the lungs of control and procysteine-treated mice on day 2. A similar trend in the production of these cytokines in the lungs of mice treated with or without procysteine was also observed on day 3 (IL-6, 178.8 ± 29.0 versus 157.3 ± 20.9 pg/mL; TNF-α, 188.2 ± 21.5 versus 224.5 ± 30.3 pg/mL).

**Improved phagocytosis in alveolar macrophages treated with procysteine**

To test whether procysteine improved phagocytosis in alveolar macrophages, we collected macrophages from the BALF of mice treated with or without procysteine and kept in hypoxic conditions. The phagocytosis of FITC-labelled beads was then analysed by fluorescence microscopy (Figure 3). The mean uptake of labelled beads by alveolar macrophages from mice treated with procysteine was 3.12 ± 0.30 beads/cell, whereas the uptake of beads by untreated macrophages was 1.81 ± 0.22 beads/cell (P < 0.001). The mean uptake of labelled beads by alveolar macrophages in control mice was 4.05 ± 0.36 beads/cell. These results suggest that procysteine increases the phagocytic activity of alveolar macrophages in mice kept in hypoxic conditions.

**Discussion**

In previous studies, 8% of the infections or clinical cases of VAP have been associated with Acinetobacter species. The increasing development of multiple antimicrobial resistance in this pathogen has severely restricted the therapeutic options available for infected patients. Our previous reports showed that hypoxia exaggerates Legionella pneumonia resulting from apoptosis due to accelerated hyperoxia-induced ALI in a mouse model. A functional in vitro experiment revealed that exposure of primary murine alveolar epithelial cells to Legionella in conjunction with hyperoxia accelerated apoptosis and loss of barrier function. In this study, we demonstrated the interaction between hyperoxia and Acinetobacter infection in a murine model of pneumonia. Although apoptotic lung injury was undetectable in this study (data not shown), improved phagocytic activity of alveolar macrophages isolated from mice kept in hyperoxic conditions was observed. Morrow et al. reported that the phagocytic activity of RAW 264.7 cells and alveolar macrophages from C57BL/6 mice was impaired in hypoxic conditions. Baleire et al. also reported that alveolar macrophage phagocytosis and killing of Klebsiella pneumoniae were significantly reduced in vivo under hyperoxic conditions. Although the surface expression of Toll-like receptor 4 on alveolar macrophages was significantly decreased following in vivo hyperoxia, the detailed mechanism has not been described. Therefore, a better understanding of the mechanisms underlying Acinetobacter pneumonia accelerated by hyperoxia is desirable.

Uncontrolled ROS production contributes to protein and DNA injury, inflammation, tissue damage and subsequent cellular apoptosis. Antioxidants are therapeutically effective against ROS damage. Antioxidant defence systems co-evolved with aerobic metabolism to counteract oxidative damage caused by oxygen free radicals. Although supplements of vitamins C and E were applied through protocols aimed at preventing diseases such as atherosclerosis, pre-eclampsia or hypertension, the efficacy of these antioxidants remains controversial in clinical trials and meta-analysis. Bernard et al. reported that repletion of glutathione (GSH) might safely be accomplished with procysteine and N-acetylcycteine in patients with ALI and ARDS. Procysteine is a GSH precursor that links GSH synthesis and metabolism. GSH performs numerous important functions, including protection against oxidative stress. In vitro experiments indicate that procysteine increases phagocytic activity in macrophages impaired by hyperoxic conditions. To confirm the effect of procysteine on Acinetobacter pneumonia in mice, we examined the survival rate of Acinetobacter-infected mice kept in hyperoxic conditions. Although all mice infected with Acinetobacter under hyperoxic conditions died by day 3, procysteine treatment significantly improved survival. We also observed that procysteine treatment decreased the lung bacterial burden on days 2 and 3. However, there were no significant differences in both inflammatory cytokines (IL-6 and TNF-α) and histone DNA and GSH between Acinetobacter pneumonia mice kept in normoxic and hyperoxic conditions (data not shown). Our results suggest that the improved survival of infected mice kept in hyperoxic conditions following administration of procysteine may be due, at least in part, to the increased phagocytic activity of macrophages in the lung. Further research into the role of procysteine against hyperoxia-induced dysfunction in the lung is warranted.

A clinical study has shown that the number of days of ALI was decreased and there was also a significant increase in the cardiac index in procysteine treatment groups (189 mg/kg/day for 10 days). However, there was no difference in mortality between the placebo- and procysteine-treated groups. Morris et al. reported that procysteine (210 mg/kg/day for 14 days) did not improve survival or reduce ventilator time in patients with ARDS. These results suggest that the appropriate dosage and administration period of procysteine may be critical factors in ARDS patients. In the present study, we established Acinetobacter murine pneumonia in hyperoxic conditions and then evaluated...
the dose- and duration-dependent effects of procysteine on lethality in this model. We determined the appropriate timing (once daily for 4 days starting on day 0) and dosage (160 mg/kg/day) of procysteine treatment in the Acinetobacter VAP model. Lee et al.\textsuperscript{12} demonstrated that procysteine mediated significant protective effects on cisplatin-induced renal injury at a dose of 80 mg/kg. The discrepancy between dosages may result from the different mouse strains employed (C57BL/6 and BALB/c). Notably, in non-infected mice in hyperoxic conditions for 120 h, a relatively high mortality was observed in the procysteine-treated group (160 mg/kg/day for 6 days) compared with the untreated group (data not shown). This result suggests that the long-term administration of procysteine may have an adverse effect on survival in this model. Therefore, larger clinical studies and animal models are required to confirm the efficacy of procysteine.

In conclusion, the present results suggest that hyperoxia exaggerates Acinetobacter pneumonia in our mouse model and that procysteine improves the phagocytic activity of alveolar macrophages in mice kept in hyperoxic conditions. In addition, our study indicated that the appropriate timing and period of administration of procysteine might be critical factors for treating hyperoxia. Our data may lead to an improvement in the clinical outcomes of patients with VAP. However, further study of the efficacy of combination therapy involving procysteine and antibiotics is required.

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**Transparency declarations**

None to declare.

**References**


