The protective effects of Ambroxol in *Pseudomonas aeruginosa*-induced pneumonia in rats

Xiwen Gao[1], Yi Huang[2], Yipin Han[2], Chun-xue Bai[3], Guifang Wang[3]

[1]Department of Internal Medicine, Minhang District Central Hospital, Ruijin Hospital Group, Shanghai, PR China
[2]Department of Pulmonary Medicine, Changhai Hospital, Second Military Medical University, Shanghai, PR China
[3]Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, Shanghai, PR China

Submitted: 29 August 2010
Accepted: 8 February 2011

Arch Med Sci 2011; 7, 3: 405-413
DOI: 10.5114/aoms.2011.23403
Copyright © 2011 Termedia & Banach

Corresponding author:
Guifang Wang MD, PhD
Department of Pulmonary Medicine
Zhongshan Hospital
Fudan University
Shanghai, PR China
Phone: 86 21 6404 1990 2445
Fax: +86 21 5496 1729
E-mail:
Wang_guifang08@126.com

Abstract

Introduction: To evaluate the effect of Ambroxol on the pulmonary surfactant (PS) in rat pneumonia induced by *Pseudomonas aeruginosa* (PA).

Material and methods: The pneumonic rats were obtained by injecting ATCC27853 intratracheally. One hundred and twenty SD rats were randomized into four groups: normal saline and Ambroxol was injected intraperitoneally following PA challenge in the PA/NS and PA/AM group; the other two groups were NS/AM and NS/NS. The wet/dry weight ratio (W/D), and pathological changes were assayed. Total proteins (TP), total phospholipid (TPL), and dipalmitoylphosphatidylcholine (DPPC) in bronchial alveolar lavage fluid (BALF) were analysed. Some BALF was cultured for colony counts. Ultrastructural change of the lung was observed by electron microscopy.

Results: The W/D ratio in the PA/AM group was lower than that in the PA/NS group; both were higher than that in the NS/NS group ($p < 0.05$). There were more neutrophils in the PA/NS group than in the PA/AM group ($p < 0.05$), and more in the PA/AM group than in the NS/NS group ($p < 0.05$). The ratio of DSPC/TPL and DSPC/TP in the BALF in PA/NS group was lower than that in the PA/AM group; DSPC/TPL and DSPC/TP ratios also increased in the NS/AM group. The PA colony numbers in the PA/AM group were lower than in the PA/NS group ($p > 0.05$). In the PA/NS group, vacuolation occurred in the lamellar body of alveolar type 2 cells (AT2) and the PS layer was rough and broken in some areas. In the PA/AM group, the degree of vacuolation of the lamellar body was less than in the PA/NS group.

Conclusions: Ambroxol could protect rats from pneumonia by improving the level of endogenous PS, especially DPPC.

Key words: pulmonary surfactant, *Pseudomonas aeruginosa*, bronchoalveolar lavage, pneumonia, Ambroxol.

Introduction

Pseudomonas aeruginosa (PA) is the one of the most common pathogens involved in nosocomial pneumonia and is responsible for both high mortality and morbidity in critically ill patients [1]. Presently, treatment for this disorder consists primarily of antibiotic administration, but typical antibiotic combinations have little effect on patient morbidity and mortality with the rising resistance rate to many drugs [2, 3]. This is derived from

its multiple mechanisms of innate and acquired resistant-antibiotics [4-6], especially developing drug-induced resistance. Therefore other methods apart from antibiotics should be investigated to manage this disorder.

Studies on bronchoalveolar lavage fluid (BALF) obtained from patients with both Gram-positive and -negative pneumonia have demonstrated alterations in the endogenous surfactant system [7-9]. This is partially due to the degradation of bacterial content to the surfactant, for example, the combination of phospholipase C and lipase from PA was reported to result in degradation of dipalmitoylphosphatidylcholine (DPPC), producing palmitic acid and dipalmitoylglycerol, though lipase alone produced no degradation [10]. Further evidence suggests that surfactant may play a role in the lung dysfunction associated with pneumonia stems from the observed surfactant alterations in patients with acute respiratory distress syndrome (ARDS), which is associated with similar alterations in lung mechanics and the surfactant system. Some studies have shown that these changes contribute to the lung dysfunction observed in ARDS and exogenous surfactant has shown promise as a therapeutic modality in these patients and animal models such as rabbits and rats [11-14].

But, the elements of exogenous pulmonary surfactant are different from one another; for example, Exosurf is a synthetic pulmonary surfactant [15], Survanta is a natural bovine surfactant extract (including SP-B, SP-C, not SP-A) [15], Curosurf is a porcine surfactant extract (SP-B, SP-C) [16]. Therefore there have been some difficulties to contrast the efficacy between any two medications and set up a standard formula of PS administration. But Ambroxol, a mucolytic agent, exhibits not only antioxidant and anti-inflammatory properties with reduction of the release of inflammatory cytokines from bronchoalveolar macrophages, monocytes and granulocytes [17], but also, it is found to increase alveolar type II cell (AT2)-secreted surfactant proteins [18]. Therefore if used as the actor of a surfactant, it can be administered easily and contrasted the efficacy. There is now ample evidence that Ambroxol is a very potent inducer of surfactant synthesis in young and adult organisms and in alveolar type II cells from different animals [19]. This study was performed to use Ambroxol for treating pneumonia induced by PA and to provide some support for further clinical use.

Material and methods

Materials

Animals

Protocols for animal care and experimental management were approved by the Fudan University's Scientific Committee. Healthy adult SD rats (~220-240 g) were anaesthetized with 1% sodium pentobarbital intraperitoneally at a dose of 50 mg/kg.

Bacteria

ATCC27853 is a standard line of PA obtained from ATCC. *Pseudomonas aeruginosa* cultured in broth for 18 h were diluted to 2 McFarland with normal saline (6×10^8 CFU/ml, 1 McFarland = 3×10^8 CFU/ml).

Drug for treatment

Ambroxol hydrochloride injection (Mucosolvan^R) (Boehringer Ingelheim Co. Ltd, China), 7.5 mg/ml. Normal saline was used as the control of Ambroxol.

Methods

Animal experiment procedure

One hundred and twenty rats were divided randomly into four groups, 30 rats in each group : PA/NS group, PA/AM group, NS/AM group, NS/NS group. The rats in the PA/NS group and PA/AM group were anaesthetized with sodium pentobarbital (50 mg/kg) administered intraperitoneally. This study had two stages which contained animal infected PA and the treatment of infection. After the trachea was exposed by blunt dissection and tracheal annulations, all of them were instilled with 0.2 ml *P. aeruginosa* suspension intratracheally followed by an injection of 0.5 ml of air (2 McFarland units of ATCC27853 suspension obtained according to the former methods) to copy the pneumonia of rat models. After 6 h, these rats were administered normal saline (the volume of NS was 0.6 ml, which was equal to the volume of used Ambroxol) and Ambroxol (20 mg/kg) through the vena caudalis. Both the NS/AM group and NS/NS group were given NS (0.2 ml) intratracheally. Six h later, the rats in the NS/AM group received Ambroxol (20 mg/kg) and in the NS/NS group received normal saline 0.6 ml via a 28 G catheter respectively. Four groups were allowed to recover and ad liter.

Samples procedure

After 6 h, samples were obtained according to the research aims. Most of them were referred as follows.

1) Wet-to-dry lung weight measurements

After the rats had been sacrificed, an incision was made in the abdominal wall and the chest was then opened. In each group (five animals), a piece of lung tissue (about 2 g) from the back side of the left lower lobe was cut and the wet weight was

determined in an automatic electric balance (AP250D; Ohaus, Florham, NJ). All pieces of lung tissue were then stored at 70°C for 72 h and weighed again to obtain their dry weight for calculating the wet-to-dry weight ratio (W/D).

In another four animals in each group both lungs were fixed by vascular perfusion for histological analysis. The pulmonary vessels were lavaged with normal saline via right ventricular. The rat lungs were fixed at the pressure of 20 cm H_2O by intratracheal injection of a solution containing 2% glutaraldehyde and 1% paraformaldehyde in a 0.1 M phosphate buffer solution. The lungs were then prepared for sectioning using standard techniques. Sections were stained with haematoxylin-eosin. Polymorphonuclear cells (PMN) were counted on each slide by a pathologist not knowing the experiment project and repeated three times.

2) BALF

The volume of normal saline each animal needed was counted according to the dose of 40 ml/kg. Each rat needed 8.8-10.6 ml and was lavaged according to the ratio of 4 : 3 : 3 three times. More than 85% of the instilled liquid was collected from each rat, and collected BALF was pooled and its total volume was recorded. Total 0.1 ml BALF was serially diluted and spread on blood agar plates for calculation of viable colony-forming units (CFU) of PA. After culture for 24 h at 37°C, all CFU were counted. All other BALF was immediately centrifuged for 3000 rpm lasting for 10 min to remove the cell debris, the supernatant was stored at –20°C for biochemical analysis. Samples for EM processed: all procedures for samples are described in the supplementary materials.

3) Chemical analysis of bronchoalveolar lavage fluid and total protein

All analysis methods are presented in Appendixes 1 and 2.

Statistical analysis

Statistics were conducted using SPSS software (SPSS, Chicago, IL) and the results are presented as means ± SD. One-way analysis of variance (ANOVA) procedures and post hoc analysis (LSD, significance level set at $p < 0.05$) were used for multiple comparisons.

Results

The appearance of rats inoculated with PA intratracheally and the weight ratio of wet/dry of the rat lungs of each group

After being inoculated with PA intratracheally from one to 3 h, all rats in both the PA/NS group and PA/AM group showed trembling, cyanosis, and some blood-tinged foams out of mouths and noses. All these rats breathed faster than before. Even one or two rats twitched with their hair being wet. Six h after being injected with Ambroxol, the rats in the PA/NS group acted just like before, but animals in the PA/AM group calmed down gradually.

All animals administered NS and Ambroxol respectively in the first stage, which were referred to NS/AM and NS/NS groups, did not show uncomfortable appearance.

To assay the water in the lungs of each group, the wet/dry weight ratio (W/D) was calculated. It was found that lung W/D increased significantly in the PA/NS group, which was 8.58 ±0.39. This ratio (7.47 ±0.22) in the PA/AM group was lower than that in the PA/NS group ($p < 0.05$), but both of them were significantly higher than that in both NS/AM and NS/NS groups. There was no statistical difference between NS/AM and NS/NS groups (Figure 1).

Pathological appearance

Macropathologically, the lungs weighed and enlarged in all of the PA-challenged rats (PA/NS, PA/AM group) much more than in NSor AM-challenged ones (NS/AM, NS/NS groups). The surfaces of the lungs from the PA-challenged rats were roughened with some purulent spots or pustules whose diameters were about 1-3 mm. The lungs of the PA/NS group were cut into several pieces; pink fluid was squeezed. The pulmonary pathological changes showed a decreasing trend of severity from the PA/NS to PA/AM group, while the lungs from the rats of the NS/AM and NS/NS group showed no changes.

Pulmonary pathological study revealed striking contrasts between the PA/NS group and the PA/AM group. In the lung of rats from the PA/NS group, there was bronchial mucosal epithelial cell exfoliation, hyperaemia and oedema seen in the bronchial submucosal layer with inflammatory cell infiltration, most of which were neutrophils, a few lymphocytes

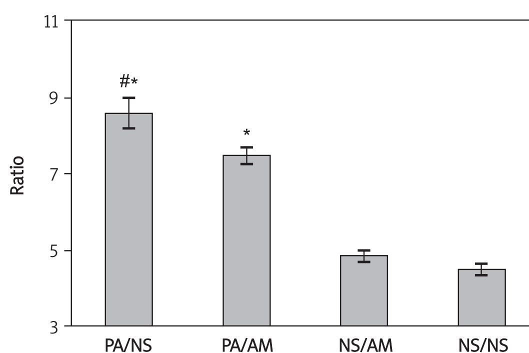

Figure 1. Wet/dry ratio of the lung of each group: wet/dry ratio of lung in PA/NS group was higher than that in PA/AM group ($p < 0.05$); both were higher than that in NS/AM and NS/NS group ($p < 0.01$), #NS/AM and NS/NS group ($p < 0.05$) and PA /AM group ($p < 0.05$), *any other group ($p < 0.05$)

and macrophages. The alveolar walls were thickened with telangiectasia and congestive capillaries. A large amount of neutrophils and erythrocytes filled in the alveoli in areas that developed pustules. Alveolar septa ruptured in some portions of the lung due to compensatory alveolar ectasia. Compared with the PA/NS group, alveolar telangiectasia and congestive capillaries in the PA/AM group attenuated, where a scanty oedema fluid and lymphocytes were scattered in the alveolar cavities. Although some neutrophils and macrophages were found, they were far fewer than those in the PA/NS group. There were few inflammatory cells infiltrated in the lungs from both the NS/NS group and AM/NS group, with regular alveolar cavities.

The inflammation of the lungs in each group was consistent with the semi-quantitative results. The neutrophils in high power were seen as a sign of inflammatory reaction. The inflammatory reaction in the PA/NS group was slightly more prominent than that in the PA/AM group, but the latter was considerably less than that in the NS/NS and AM/NS group. The PMN number per high field in the PA/NS group was 111.4 ±14.8, in the PA/AM group 38.8 ±3.1, in the NS/AM group 6.0 ±4.4, and in the NS/NS group 4.4 ±2.6. There was a significant difference between the PA/NS group and all the other groups ($p < 0.05$, Figure 2). The other corresponding groups had no significant difference.

Analysis of the pulmonary surfactant in BALF of each group

Total protein in BALF was assayed from each group and it was found that there was higher concentration in the PA/NS group (87.0 ±8.9 mg/kg). Ambroxol reduced TP in BALF in the PA/AM group, in which it was 81.4 ±7.8 mg/kg, TP in NS/AM group was 39.4 ±8.6 mg/kg, and in the NS/NS group was 36.6 ±6.1 mg/kg (Figure 3, $p < 0.05$, among any two groups but not for NS/AM group).

The level of DPPC/TPL and DPPC/TP in the PA/NS group was 38.6 ±11.0% and 19.1 ±6.9 µg/mg respectively, which was lower than that in the NS/NS group (49.6 ±5.4% and 59.8 ±14.7 µg/mg respectively) ($p < 0.05$). However, TP in BALF in the PA/NS group was higher than that in the NS/NS group with no change of concentration of TPL appearing in the PA/NS group. And there was no significant difference of TPL between any two groups (4.6 ±1.7 mg/kg for PA/NS group, 4.7 ±0.5 mg/kg for PA/AM group, 4.1 ±1.5 mg/kg for NS/AM group and 4.4 ±1.1 mg/kg for NS/NS group respectively, $p > 0.05$ for all) (Figure 4).

The level of DPPC/TPL and DPPC/TP in the PA/AM group elevated significantly compared to the PA/NS group (65.7 ±7.8% and 38.6 ±11.0% respectively, $p < 0.05$). The concentration of DPPC/TP in BALF in the PA/AM group elevated more than that in the PA/NS group (38.6 ±10.2 µg/mg and 19.1 ±6.9 µg/mg respectively, $p < 0.05$), but there was no change in the concentration of TP and TPL (Figure 4).

And also, it was found that DPPC/TPL concentration in the NS/AM group was higher than that in the NS/NS group (68.3 ±8.4% and 38.6 ±11.0% respectively, $p < 0.05$), with the level of DPPC/TP in the NS/AM group being higher than that in the NS/NS group, which may indicate that Ambroxol could increase the biosynthesis of PS in the normal rat lung (Figure 4).

Colony of bacteria in BALF of each group

There were no bacteria growing in media from BALF of both NS/AM and NS/NS groups. And there were many bacteria growing in media from BALF of

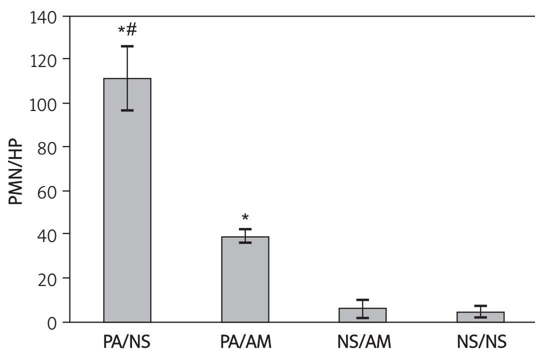

Figure 2. Semi-quantitative analysis of PMNs per high power field under the light microscope. After pathological slides were prepared, analysis of PMN was performed by a pathologist not knowing the experiment project. PMN numbers per high power field in PA/NS group were 111.4 ±14.8, in PA/AM group 38.8 ±3.1, in NS/AM group 6.0 ±4.4, and in NS/NS group 4.4 ±2.6. There was a significant difference between PA/NS group and all other groups *vs. NS/NS group (p < 0.05), #vs. PA/AM group ($p < 0.05$)

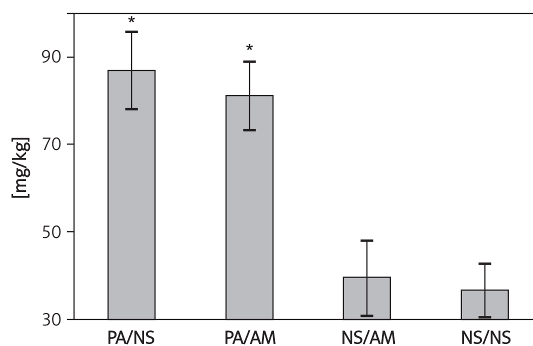

Figure 3. Total protein (TP) in BALF from lungs in each group. TP in PA/NS group was higher than that in NS/NS group (87.0 ±8.9 mg/kg and 36.6 ±6.1 mg/kg respectively, $p < 0.05$), both were higher than that in NS/AM and NS/NS group ($p < 0.01$), *vs. NS/NS group ($p < 0.05$)

both PA/NS and PA/AM groups, which were identified as PA, whose biological features were just like the bacteria inoculated intratracheally. There were 10.35 ±2.10 (10^4 CFU/ml) and 8.02 ±3.2 (10^4 CFU/ml) respectively in PA/NS and PA/AM groups, but there was no statistical difference between the two groups ($p > 0.05$) (Figures 5 A-C).

Performance of lungs under electron microscopy

The normal ultramicrostructure of the lamellar body in alveolar type II cells (AT2) and tubular myelin (TM) for exocytosis being intruded into the alveolar cavity could be found in both NS/NS and NS/AM groups (Figure 6 E). The PS layer on the surface of alveoli was homogeneous in both groups (Figure 6 F). But in the PA/NS group, vacuolation occurred in the lamellar body of AT2. The lattice and other types of tubular myelin exfoliated into the alveolar cavity (Figures 6 A, B). The PS layer was rough and was broken in some areas. In the PA/AM group, the degree of vacuolation of the lamellar body had more tendency to recovery than those in the PA/NS group and the PS layer was more perfectly distributed than the PA/NS group (Figures 6 C, D).

Discussion

Here, by injecting PA ATCC27853 intratracheally, rat pneumonia models were produced. After being inoculated for 3 h, all rats challenged with PA showed trembling, dyspnoea and hair-wet. The pathological lungs weighed and enlarged in all of the PA-challenged rats. Lung surfaces were rough with some purulent spots or pustules. A pink water-like fluid flowed from its cut cross-sectional surface

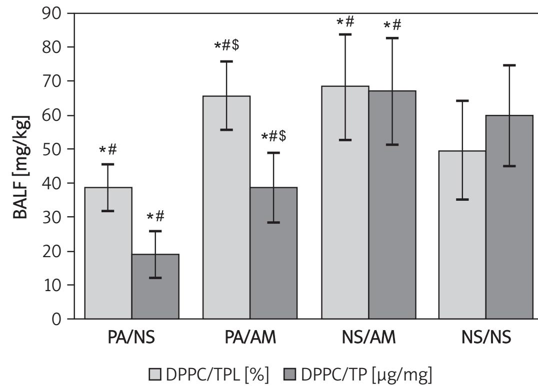

Figure 4. Analysis of contents of BALF in lungs from each group. Level of DPPC/TPL and DPPC/TP in PA/AM group elevated significantly compared to PA/NS group (65.7 ±7.8% and 38.6 ±11.0% respectively, $p < 0.05$). Concentration of DPPC/TP in BALF in PA/AM group elevated more than that in PA/NS group (38.6 ±10.2 μg/mg and 19.1 ±6.9 μg/mg respectively, $p < 0.05$). DPPC/TPL concentration in NS/AM group was higher than that in NS/NS group (68.3 ±8.4% and 38.6 ±11.0% respectively, $p < 0.05$), with the level of DPPC/TP in NS/AM group being higher than that in NS/NS group

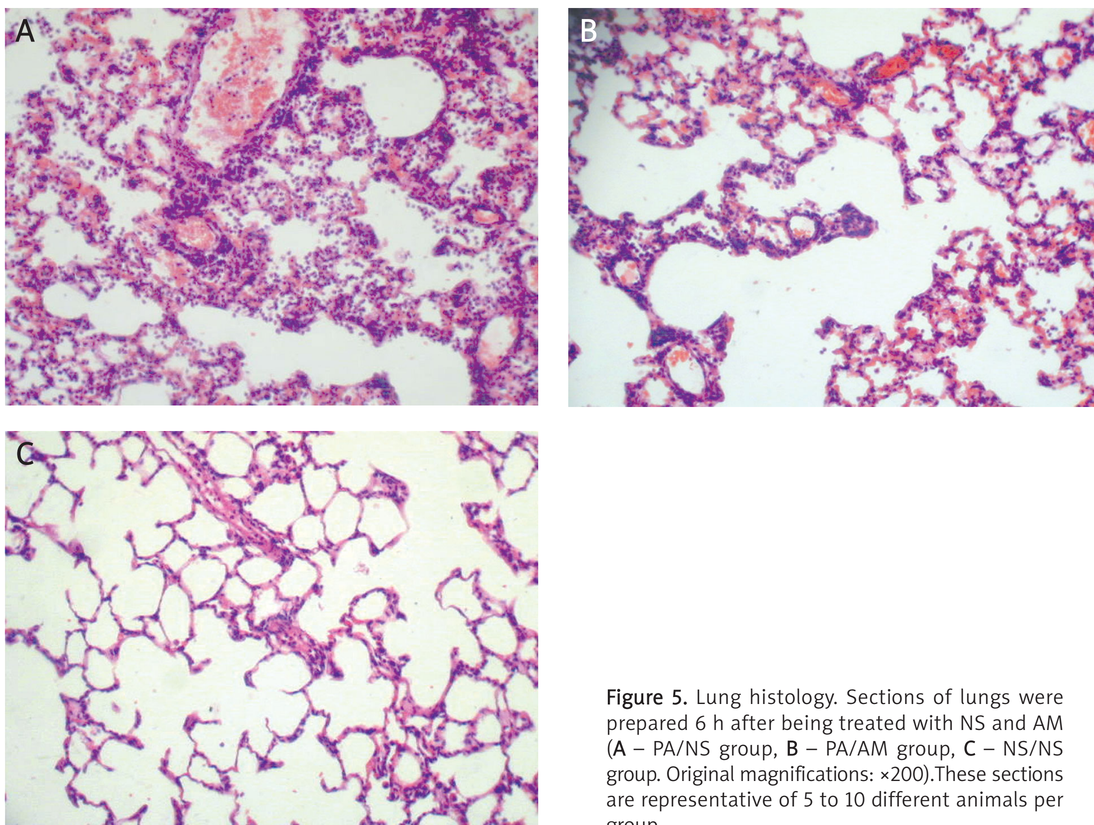

Figure 5. Lung histology. Sections of lungs were prepared 6 h after being treated with NS and AM (**A** – PA/NS group, **B** – PA/AM group, **C** – NS/NS group. Original magnifications: ×200).These sections are representative of 5 to 10 different animals per group

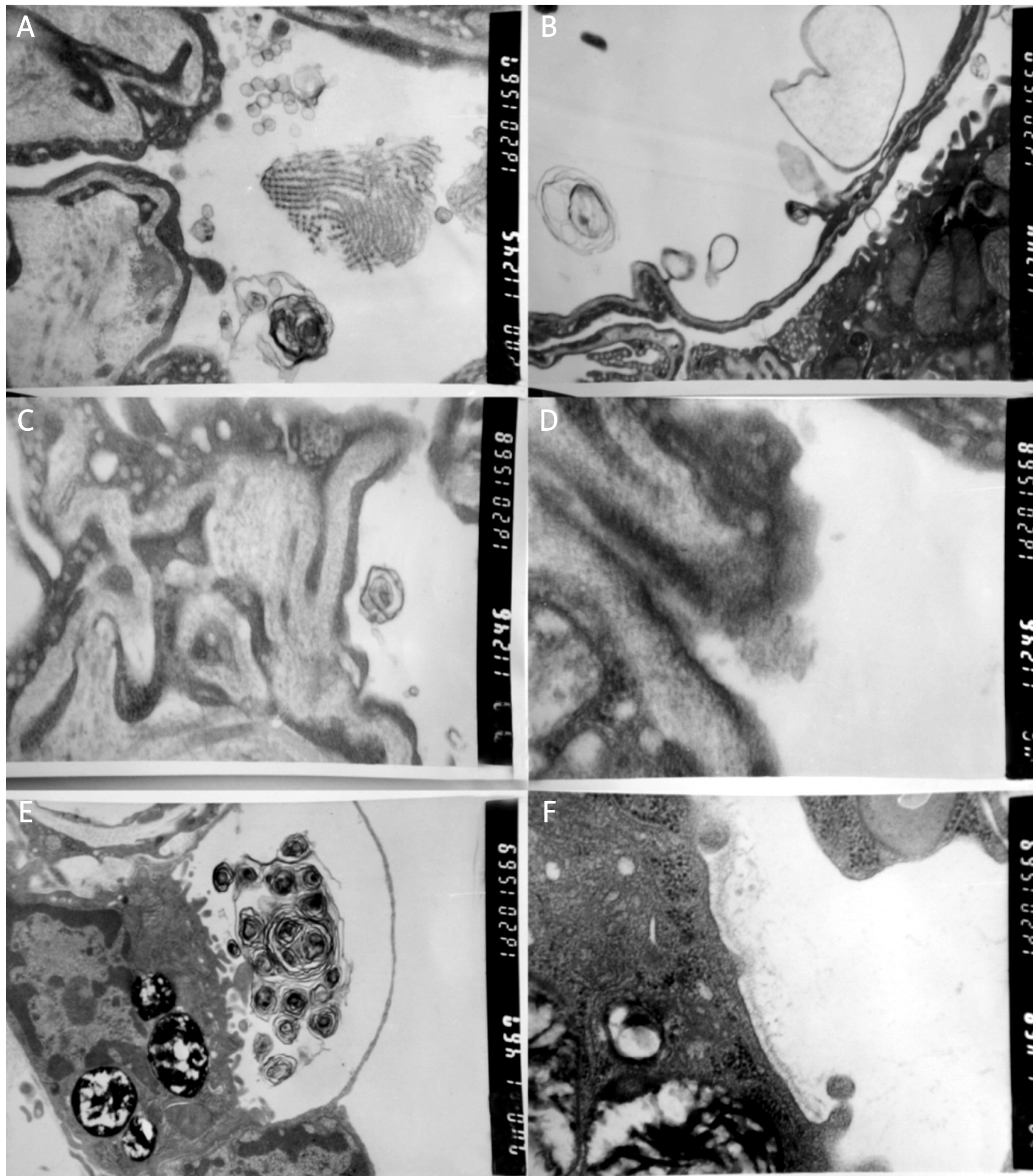

Figure 6. Electron microscopy. Sections of lungs were prepared 6 h after being treated with NS and AM and the procedure was according to the supplementary materials online. Normal ultramicrostructure of lamellar body in alveolar type II cells (AT2) and tubular myelin (TM) for exocytosis being intruded into alveolar cavity all could be found in both NS/NS and NS/AM group (**E**). The PS layer on the surface of alveoli was uniform in these two groups (**F**). But in PA/NS group, vacuolation occurred in lamellar body of AT2. Lattice and other types of tubular myelin exfoliated into the alveolar cavity (**A**, **B**). PS layer was rough and was broken in some areas. In PA/AM group, degree of vacuolation of lamellar bodies had more tendency to recovery than those in PA/NS group and PS layer was more perfectly distributed than PA/NS group (**C**, **D**)

when squeezed. And also the lungs from the rats challenged with PA showed higher W/D ratio than that with NS and Ambroxol. More than that, PA were cultured from BALF obtained from rats challenged with PA, and their biological features were identical to the inoculated ones. Under light microscopy, inflammatory cells infiltrated the lungs and most of them were neutrophils. All this indicated that the pneumonia models succeeded.

To assay the BALF content, it was found that TP in BALF from rats in the PA/NS group increased, while DPPC/TPL, DPPC/TP decreased, which may indicate that DPPC in PS was relatively lowered. This work presented results just like those obtained by Wanderzwan *et al.* [23]; they found that the content of TPL, DPPC and large surfactant aggregates (LSA) decreased. Together these assays of the level of DPPC/TPL, DPPC/TP in BALF in

severe pneumonia could suggest the prognosis of pneumonia.

Now it is suggested that phospholipase secreted by bacteria may play some role to decrease PS in bacterial pneumonia. Lema *et al*. [24] found the calf lung surfactant extract hydrolysis was catalyzed by extracts (mostly phospholipase C) of the PA, particularly the non-mucoid types, which severely affected surfactant function. And they concluded that this mechanism might be the most important injury factor. Here in our study the lung in the PA/NS group under electron microscopy presented that PS liner ruptured and twisted, and even dropped into the alveolar cavities, which indicated that some concents of PA injured the PS liner. If being given Ambroxol, the extent of injury lessened.

It is well known that the PS is synthesized and secreted by AT2, most of which consists of phospholipids (PL) and up to 80% to 85%, mainly dipalmitoylated phosphatidylcholine (DPPC) and phosphatidylglyceride (PG), and acts to maintain alveolar stability. And also, PS, especially PL, can modulate the inflammatory reaction of normal and abnormal lungs, and even has bacteria-inhibiting effects [12, 25]. Among all contents of PS, SP-D plays a more important role than any other. There were some results about human pneumonia that showed decreased SP-A in patients with bacterial pneumonia [26].

From the results, it could be found that there was a large amount of infiltration in the alveolar cavity, increased W/D ratio, and also TP rise, and lower DPPC/TPL and DPPC/TP. All these combined together may indicate the deficiency or impairment of the PS layer, and this gave evidence of exogenous PS replacement. Some researchers [27] found that a large amount (~300-500 mg/kg b.w.) by intrabronscopic administration in septic shock patients caused far-reaching restoration of biochemical surfactant properties and significant improvement. With this method, gas-exchange ability rose significantly. And Protsiuk [28] used exogenous PS associated with antibiotics to treat pneumonia in children and found that the effect of two methods was much better than that of only antibiotics. Now, Ambroxol has been used for many years all over the world to manage respiratory tract disorders. This compound was originally developed as a mucolytic agent [29], but it was soon discovered that it influences the secretory apparatus of the bronchial epithelium and pulmonary alveoli. There is now ample evidence that Ambroxol is a very potent inducer of surfactant synthesis in young and adult organisms and in alveolar type II cells from different animal species [30]. The mechanism has not yet been understood completely. Von Vichert *et al*. [31] reported that Ambroxol in healthy rabbits increases the phospholipid content of the lung.

This study also proved by EM that Ambroxol could promote the function of PS. In rats from PA/NS group, it was found that vacuolation occurred in the lamellar body in AT2. The lattice and other types of tubular myelin exfoliated into the alveolar cavity. The PS layer was rough and was broken in some areas. In the PA/AM group, the degree of vacuolation of the lamellar body had more tendency to recovery than those in the PA/NS group and the PS layer was more perfectly distributed in the AT2 than that in the PA/NS group, and few lamellar bodies exfoliated. This result indicated that after AT2 were stimulated, the synthesis and secretion of PS also increased.

Another study indicated that Ambroxol can help to increase the local concentration of antibiotics and reduce the morbidity of infectious diseases [32, 33]. In our work, though there was no statistical difference regarding the amount of bacteria's CFU between the PA/NS group and PA/AM group ($p > 0.05$), the inflammatory infiltration of lungs in the PA/AM group was less than that in the PA/NS group. Yang *et al*. [34] suggested that Ambroxol protected mice challenged with influenza virus from lethal effects and improved the mice survival rate partly through inhibiting the releasing of cytokines such as TNF-α, IFN-γ and IL-12, and promoting the secretion of the release of suppressors of influenza virus multiplication, such as pulmonary surfactant (mostly SP-A, rising 1.5-1.7 fold), immunoglobulin (Ig)-A and IgG, etc. Therefore it may be that Ambroxol could stimulate the synthesis and secretion of exogenous PS in lungs of infected rats and exert a protective effect. Further, analysis of BALF of non-infected rats suggested that this stimulation was not limited to infected rats, which meant in normal rat lungs Ambroxol acted in the same role. Now, exogenous PS is very expensive and there is no standard about this drug. But all packages of Ambroxol are relatively economical and the usage is relatively simple, so it perhaps acts on the responsibility and aids in severe or resistant lung infection. The definite mechanism of this drug might need to be investigated.

Acknowledgments

This work was supported by Shanghai Leading Academic Discipline Project (Project Number: B115). We also appreciate Dr. Albert for his critical comments and efforts to improve the quality of the manuscript. And we thank Mr. Jiang for preparing the figures.

References

1. Fagon JY, Chastre J, Domart Y, Trouillet JL, Gibert C. Mortality due to ventilated-associated pneumonia or colonization with pseudomonas or Acinetobacter species: assessment by quantitative culture of samples obtained

by a protected specimen brush. Clin Infect Dis 1996; 23: 538-42.
2. Ye F, Zhong SP, Yuan JP. Serial five-year-surveillance of resistant-rate of Gram-negative bacilli isolated from patients' lower airway tract in respiratory intensive care unit. Anti-infect Chemother Acta 2007; 7: 367-71.
3. Combes A, Luyt CE, Fagon JY, Wolff M, Trouillet JL, Chastre J. Impact of piperacillin resistance on the outcome of Pseudomonas ventilator-associated pneumonia. Intensive Care Med 2006; 32: 1970-8.
4. Gillis RJ, White KG, Choi KH, Wagner VE, Schweizer HP, Iglewski BH. Molecular basis of azithromycin-resistant Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2005; 49: 3858-67.
5. Dean CR, Visalli MA, Projan SJ, Sum PE, Bradford PA. Efflux-mediated resistance to tigecycline (GAR-936) in Pseudomonas aeruginosa PAO1. Antimicrob Agents Chemother 2003; 47: 972-8.
6. Vanderzwan J, McCaig L, Mehta S, et al. Characterizing alterations in the pulmonary surfactant system in rat model of Pseudomonas aeruginosa pneumonia. Eur Respir J 1998; 12: 1388-96.
7. Baughman RP, Sternberg RI, Hull W, Buchsbaum JA, Whitsett J. Decreased surfactant protein A in patients with bacterial pneumonia. Am Rev Respir Dis 1993; 147: 653-7.
8. Günther A, Siebert C, Schmidt R, et al. Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. Am J Respir Crit Care Med 1996; 153: 176-84.
9. Schmidt R, Meier U, Yabut-Perez M, et al. Alteration of fatty acid profiles in different pulmonary surfactant phospholipidsin acute respiratory distress syndrome and severe pneumonia. Am J Respir Crit Care Med 2001; 163: 95-100.
10. Jaeger KE, Kharazmi A, Hoiby N. Extracellular lipase of Pseudomonas aeruginosa: biochemical characterization and effect on human neutrophils and monocyte function in vitro. Microb Pathog 1991; 10: 173-82.
11. Sun B, Herting E, Curstedt T, Robertson B. Exogenous surfactant improves lung compliance and oxygenation in adult rats with meconium aspiration. J Appl Physiol 1994; 77: 1961-71.
12. van Kaam AH, Lachmann RA, Herting E, et al. Reducing atelectasis attenuates bacterial growth and translocation in experimental pneumonia. Am J Respir Crit Care Med 2005; 172: 141.
13. Zhu GF, Sun B, Niu SF, et al. Combined surfactant therapy and inhaled nitric oxide in rabbits with oleic acid-induced acute respiratory distress syndrome. Am J Respir Crit Care Med 1998; 158: 437-43.
14. Song GW, Robertson B, Curstedt T, Gan XZ, Huang WX. Surfactant treatment in experimental Escherichia Coli pneumonia. Acta Anaesthesiol Scand 1996; 40: 1154-60.
15. Sehgal SS, Ewing CK, Richards T, Taeusch HW. Modified bovine surfactant (Survanta) versus a protein-free surfactant (Exosurf) in the treatment of respiratory distress syndrome in preterm infants: a pilot study. J Natl Med Assoc 1994; 86: 46-52.
16. Alberti A, Pettenazzo A, Enzi GB, et al. Uptake and degradation of Curosurf after tracheal administration to newborn and adult rabbits. Eur Respir J 1998; 12: 294-300.
17. Pfeifer S, Zissel G, Kienast K, Muller-Quernheim J. Reduction of cytokine release of blood and bronchoalveolar mononuclear cells by ambroxol. Eur J Med Res 1997; 2: 129-32.
18. Seifart C, Clostermann U, Seifart U, et al. Cell-specific modulation of surfactant proteins by ambroxol treatment. Toxicol Appl Pharmacol 2005; 203: 27-35.
19. Post M, Batenburg JJ, Schuurmans EAJM, Oldenborg V, van der Molen AJ, van Golde LGM. The perfused rat lung as a model for studies on the formation of surfactant and the effect of ambroxol on this process. Lung 1983; 161: 349-59.
20. Bartlett GB. Phosphorous assay in column chromatography. J Biol Chem 1959; 234: 466-71.
21. Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium tetroxide. J Lipid Res 1976; 17: 281-4.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-75.
23. Vanderzwan J, McCaig L, Mehta S, et al. Characterizing alterations in the pulmonary surfactant system in rat model of Pseudomonas aeruginosa pneumonia. Eur Respir J 1998; 12: 1388-96.
24. Lema G, Dryja D, Vargas I, Enhorning G. Pseudomonas aeruginosa from patients with cystic fibrosis affects function of pulmonary surfatant. Pediatr Res 2000; 47: 121-6.
25. Wu H, Kuzmenko A, Wan S, et al. Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. J Clin Invest 2003; 111: 1589-602.
26. Baughman RP, Stermberg RI, Hull W, Buchsbaum JA, Whitsett J. Dereased surfactant protein-A in patients with bacterial pneumonia. Am Rev Respir Dis 1993; 147: 653-7.
27. Bronchoscopic administration of bovine natural surfactant in ARDS and septic shock: impact on biophysical and biochemical surfactant properties. Eur Respir J 2002; 19: 797-804.
28. Herting E, Gefeller O, Land M, et al. Surfactant treatment of neonate with respiratory failure and group B streptococcal infection. Pediatrics 2000; 106: 957-64.
29. Grassi C, Luisetti M, De Rose V, et al. Biochemical and functional changes in bronchoalveolar parameters induced by ambroxol treatment of chronic bronchitis. In: Pulmonary surfactant system. Cosmi E, Scarpelli E (eds.). Elsevier, Rome 1983; 361-70.
30. Post M, Batenburg JJ, Schuurmans EAJM, Oldenborg V, van der Molen AJ, van Golde LGM. The perfused rat lung as a model for studies on the formation of surfactant and the effect of ambroxol on this process. Lung 1983; 161: 349-59.
31. Von Vichert P, Bavendamm U, Von Teichmann M, et al. Increased incorporation of fatty acids into phospholipids of lungs and livers of rabbits under the influence of bromexine and ambroxol. Archiv Pharmacol 1977; 297: 269-73.
32. Fraschi. Effects of a mucolytic agent on the bioavaility of antibiotics in patients with chronic respiratory disease. Curr Therap Res 1997; 8: 5.
33. Winselk. Antioxidant and anti-inflammatory properties of amboxol. Pheumology 1992; 9: 461
34. Yang B, Yao DF, Ohuchi M, et al. Ambroxol suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor levels. Eur Respir J 2002; 19: 952-8.

Appendix 1

Immediately after the last cardiac beat the tracheal perfusion at 40 cm H_2O pressure with 3 ml 25% glutaraldehyde in 0.1% phosphate buffer (pH 7.0) preceded the clamping of the trachea. The heart-lung block was sectioned and immersed in 3% glutaraldehyde for 1 h. Ten samples (5 subpleural and 5 central) measuring about 1 × 1 mm for TEM were taken from each lobe of the right lung (cranial, middle, caudal, postcaval) and from the left lung lobe. These samples were prefixed in 3% glutaraldehyde in 0.1% phosphate buffer (pH 7.0) for 3 h and washed 3 times with the same phosphate buffer. The samples were postfixed in a phosphate buffer 1% OsO_4 at 6°C for 3 h, dehydrated in a graded ethanol series, infiltrated with propylene oxide and embedded in Ladd's epon LX-112. From both edges of all blocks, 20 semiserial (interval 20 µm) semifine sections (0.5 µm) were prepared and stained with toluidine blue for light microscopic (LM) analysis. According to the LM findings (presence and number of diatoms in alveolar space and/or close to the alveolar wall, positive findings, etc.) a strike selection of the blocks (exclusion rate = 98%) was performed and 5 were analysed. From the selected samples, serial and semiserial (interval 0-40 µm) semifine sections were prepared and a step-by-step decision was made whether or not to prepare 5-10 ultrathin serial sections. The ultrathin sections were stained on grids using uranyl acetate and lead citrate. For the ultrastructural study and micrography a HITACHI H-800 electron microscope was used.

Appendix 2

Aliquots of BALF were extracted with threefold volumes of chloroform-methanol (2 : 1, vol/vol) to isolate the phospholipids in the chloroform phase. DSPC was separated from other phospholipids as described by Mason *et al*. [20]. Briefly, samples from the chloroform phase were dried under nitrogen gas, oxidized with small volume of osmium tetroxide in carbon tetrachloride for 15 min, and dried again under nitrogen, dissolved in chloroform-methanol (20 : 1, vol/vol), and passed through a neutral aluminium column. The DSPC fraction was collected by adding to the column a mobile phase of chloroform-methanol-7M ammonium hydroxide (70 : 30 : 2, vol/vol/vol). Amounts of DSPC and TPL were determined according to the methods described by Bartlett [21] and corrected by the total volume of BALF and body weight. Values for TPL are presented as milligrams per kilogram, DSPC as a percentage of the TPL (DSPC/TPL). Total proteins (TP) in BALF were measured according to the method of Lowry *et al*. [22], using bovine serum albumin as the standard, and corrected by total volume of BALF and body weight; these values are presented as milligrams per kilogram. The DSPC and TP ratio was expressed as micrograms per milligram.