Aerosolized surfactant therapy for endotoxin-induced experimental acute respiratory distress syndrome in rats

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We have compared the effects of inhalation of aerosolized surfactant on experimental acute respiratory distress syndrome. Escherichia coli endotoxin (55 (SD 20) mg kg⁻¹) was injected into the tracheas of 36 adult rats anaesthetized and mechanically ventilated with pure oxygen. When the \( P_{aO_2} \) decreased to 11.3 (3.3) kPa, the animals were randomly subjected to inhalation of aerosolized modified natural surfactant (MNS) for 0 min (control group), 30, 60, and 120 min. In the control group, \( P_{aO_2} \) remained below 12 kPa for 180 min. In the groups receiving inhalation of surfactant for 30 and 60 min, \( P_{aO_2} \) increased but decreased soon after termination of the inhalation. In contrast, \( P_{aO_2} \) of the group receiving inhalation of surfactant for 120 min continued to increase, reaching 52.1 (12.5) kPa at 180 min (\( P<0.05 \) vs control). Thus, we conclude that improvement in gas exchange as a result of inhalation of MNS depends on the duration of inhalation.

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Pulmonary surfactant, consisting of phospholipids and surfactant-associated proteins (SPs); SP-A (hydrophilic), SP-B (hydrophobic), SP-C (hydrophobic), and SP-D (hydrophilic),12 prevents atelectasis and lung oedema by reducing surface tension of the alveoli.2 3 In acute respiratory distress syndrome (ARDS), various surfactant inhibitors appear in the alveolar space.4 5 Surfactant replacement therapy has been tried in patients with ARDS to restore surfactant function,6–8 resulting in short-term improvement in gas exchange.7,8 The replacement technique with bolus intra-tracheal instillation may present difficulties for early treatment of ARDS, as it may cause circulatory instability.9,10 On the other hand, inhalation of aerosolized surfactant can be used for such patients, as this procedure did not change arterial pressure in our previous study using rats with experimental ARDS caused by endotoxin.10 We found, however, that the therapeutic effects of inhalation of aerosolized surfactant for 60 min were inferior to those of intra-tracheal instillation of surfactant (100 mg kg⁻¹). In the present study, we examined whether prolonged inhalation of aerosolized surfactant can improve \( P_{aO_2} \) in the same experimental ARDS model.

Methods

Preparation of surfactant

A modified natural surfactant (MNS) was isolated from the lungs of recently slaughtered pigs. The alveolar lavage fluid was centrifuged at 150 g for 10 min to remove cell debris. The supernatant was further centrifuged at 2000 g for 60 min to obtain pellets of crude surfactant. This crude surfactant was purified by extraction with chloroform-methanol (2:1, v:v) and acetone precipitation.11 This surfactant consisted of 98% phospholipids (by weight), 1% other lipids, and 1% hydrophobic SPs (SP-B and SP-C).11 It was reported that this MNS was effective for treatment of babies suffering from respiratory distress syndrome12 and that it increased lung compliance of immature newborn rabbit to a similar extent as a porcine surfactant obtained with liquid–gel chromatography.13
MNS was suspended in normal saline at a concentration of 25 mg ml$^{-1}$.

**Main procedure**

With the approval of the Animal Care Committee of the Kanazawa University School of Medicine, 36 male Wistar rats weighing between 320 and 440 g were anaesthetized with an i.p. injection of pentobarbital sodium (30 mg kg$^{-1}$), and the lungs intubated through a tracheotomy. One femoral artery was cannulated to allow monitoring of arterial pressure and removal of blood samples. Lactated Ringer’s solution containing a neuromuscular blocking agent (0.02 mg ml$^{-1}$ pancuronium bromide) was continuously infused at a rate of 10 ml kg$^{-1}$ h$^{-1}$ through one femoral vein. Pentobarbital sodium (a single dose of 15 mg kg$^{-1}$) was injected into the peritoneal cavity when additional anaesthesia was necessary.

The experiment was always performed concurrently on three rats, which made up one set, each rat being connected in parallel to a pressure-controlled ventilator (Servo 900B, Siemens-Elema, Solna, Sweden) delivering 100% oxygen at a frequency of 40 breaths min$^{-1}$ with an inspiration time of 50%, and a fixed peak inspiratory pressure of 25 cm H$_2$O. At the beginning of the experiment, the positive end-expiratory pressure (PEEP) was set at 7.5 cm H$_2$O with the aid of a variable resistor (E037E, Siemens-Elema). After registration of the baseline value for arterial blood gases, PEEP was reduced to 0 cm H$_2$O, and 40 mg kg$^{-1}$ of endotoxin (E. coli O111:B4, Difco Laboratories, Detroit, MI, USA), suspended in normal saline (20 mg ml$^{-1}$), was injected into the lungs through the tracheal tube over 1 s. Arterial blood samples were analysed for $P_{aO_2}$ every 30 min. The same dose was re-administered to an individual rat whose $P_{aO_2}$ was higher than 53 kPa 30 min after the first injection.

All three rats in the set were ventilated with these ventilatory settings until $P_{aO_2}$ decreased to less than 27 kPa; this oxygen index value was chosen based on the criteria for ARDS patients$^{1,14}$ Then, the PEEP was increased in increments of 2.5 cm H$_2$O. Sequences of increment in PEEP and ventilation were repeated until the $P_{aO_2}$ decreased to less than 27 kPa despite a PEEP of 7.5 cm H$_2$O. Then, lung oedema fluid in the airway was removed by tracheal suction with a fine catheter. When the $P_{aO_2}$ values of all three rats in a set remained at less than 27 kPa 15 min after tracheal suctioning, experimental ARDS was considered to be fully developed. One rat in the set was randomly assigned to a control group and did not receive any aerosol into the airway. The other two rats were randomly assigned to two of three aerosolized surfactant groups as described below.

Rats in the AS-30, AS-60, and AS-120 groups underwent inhalation of aerosolized MNS for 30, 60, and 120 min, respectively. For this purpose, the rats were connected to an inhalation circuit constructed as a branch circuit, to which the ventilator delivered oxygen under the same conditions as for the main circuit.$^{15}$ An ultrasonic nebulizer (NE-U06, Omron, Tokyo, Japan), with its blowing system shut off, was positioned 7 cm proximal to the tracheal tube. A 3-ml aliquot of the MNS suspension was placed in the reservoir of the nebulizer, which produced an aerosol (mean droplet diameter, 3.4 (SD 1.2) μm) by means of ultrasound (2.4 MHz, 30 W).$^{15}$ The moment when the aerosol inhalation was initiated was termed the ‘starting point’. Another 3 ml of the fresh MNS suspension was added to the reservoir every 15 min. After completion of the inhalation, the rats were reconnected to the original ventilatory circuit. Arterial blood samples were then evaluated sequentially for $P_{aO_2}$, $P_{aCO_2}$, and base excess (BE) using a conventional analyser (ABL-520, Radiometer, Copenhagen, Denmark) until 180 min after the starting point. Tracheal suction was performed 5 min before each blood gas analysis. A fixed volume of 0.5 ml of sodium bicarbonate (1 Eq litre$^{-1}$) was administered for correction of metabolic acidosis after each blood gas analysis if BE was lower than −5 mEq litre$^{-1}$.

At the end of the experiment (i.e. 180 min after the starting point), a sublethal dose of pentobarbital was administered, after which the tracheal tube was plugged to cause absorption atelectasis followed by death. Next, the thorax was opened and all rats were connected in parallel to an apparatus for static pressure-volume recordings of the lung.$^{16}$ Airway pressure was raised in a stepwise manner from 0 to 30 cm H$_2$O, and then lowered, also in a stepwise manner, to 0 cm H$_2$O, with a 1-min period of stress relaxation at each 5-cm H$_2$O level. These volume measurements were corrected for gas compression.

**Additional procedure**

To assess the distribution of aerosolized MNS in the lungs, an additional experiment was performed on three pairs of lung-lavaged rats.$^{17}$ The rats were mechanically ventilated with a peak inspiratory pressure of 25 cm H$_2$O and a PEEP of 7.5 cm H$_2$O using pure oxygen, and underwent lung-lavage with normal saline (40 ml kg$^{-1}$, 37°C) six times. One rat of the pair was ventilated for 60 min without administration of any aerosolized MNS, while the other rat of the pair was subjected to inhalation of aerosolized MNS for 60 min in the same manner as described in the main procedure. Rats were then killed and their thorax opened. The lungs were expanded at an airway pressure of 10 cm H$_2$O, and were fixed by immersion of the whole body in a formalin solution (10%) for 3 h. The lungs were then removed and further fixed in formalin for several weeks.

Lung sections were examined for localization of SP-B with the aid of monoclonal mouse anti-SP-B antibody (8B5E)$^{18}$ This antibody binds with SP-B derived from both rat and pig so that both endogenous and exogenous
surfactants could be detected. In brief, the lung sections were first counterstained with Meyer’s haematoxylin and intrinsic peroxidase activity was inhibited by means of 0.3% hydrogen peroxide in methanol for 30 min. Next, they were incubated with anti-SP-B antibody in phosphate-buffered saline (PBS) for 30 min after blocking non-specific binding sites with 5% bovine serum albumin in PBS. The sections were immersed in biotinylated anti-mouse IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA, USA) and then in a solution of avidin-biotinylated horseradish peroxidase complex (VECTASTAIN ABC kit, Vector Laboratories). The final peroxidase reaction was performed with 0.05% diaminobenzidine-tetrahydrochloride and 0.01% hydrogen peroxide. The sections serving as controls were treated in the same manner but without primary antibody.

**Statistical analysis**

Data are presented as mean (SD). Differences in survival rates and the proportion of rats that needed second injections of endotoxin were assessed with Fisher’s exact test. Other numerical data were assessed by means of one- or two-way analysis of variance, modified for repeated measures, using the SuperANOVA software applications (Abacus Concepts, Berkeley, CA, USA) for the Macintosh computer (Apple Computer, Cupertino, CA, USA). When inter- and/or intra-group differences occurred, they were further analysed with contrasts. *P*<0.05 was considered significant.

**Results**

**Main procedure**

Averaged body weight was 375 (28) g with no significant inter-group differences. Rats given endotoxin twice accounted for 33% (four of twelve rats) of the control group, 50% (four of eight) of the AS-30 group, 27% (three of eight) of the AS-60 group, and 27% (three of eight) of the AS-120 group. The administered dose of endotoxin was 55 (20) mg kg⁻¹ with no significant differences among the four groups (Table 1). Elapsed time between the first injection of endotoxin and the starting point for all rats was 184 (81) min with no significant inter-group differences. In all rats arterial pressure decreased after injection of endotoxin. A total of 12 rats died by 180 min after the starting point, and mortality rate of the four groups ranged between 25 and 42% with no significant inter-group differences (Table 1). All rats that died in the control and AS-30 groups suffered from both hypoxaemia and hypotension. In the AS-60 group, one rat died of both hypoxaemia and hypotension, and one hypotension only. In contrast, two rats in the AS-120 group died of hypotension, although *P*<O₂ was maintained above 27 kPa.

*P*<O₂ of all rats was 66.0 (8.1) kPa during the baseline period, and decreased to 11.3 (3.3) kPa in the pre-starting period without significant inter-group differences. Changes in *P*<O₂ are shown in Figure 1A. The reduced *P*<O₂ of the control group remained less than 12 kPa throughout the observation period (180 min after the starting point). Thirty

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**Table 1** Body weight, endotoxin dose, and rat mortality (mean (SD)). Elapsed time: time between the first injection of endotoxin and the starting point (=aerosol initiation)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Endotoxin (mg kg⁻¹)</th>
<th>Elapsed time (min)</th>
<th>Number of dead rats (mortality rate, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>375 (31)</td>
<td>53 (20)</td>
<td>184 (83)</td>
<td>5 (42)</td>
</tr>
<tr>
<td>AS-30</td>
<td>8</td>
<td>383 (32)</td>
<td>60 (21)</td>
<td>177 (81)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>AS-60</td>
<td>8</td>
<td>378 (27)</td>
<td>55 (21)</td>
<td>191 (89)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>AS-120</td>
<td>8</td>
<td>363 (17)</td>
<td>55 (21)</td>
<td>183 (85)</td>
<td>2 (25)</td>
</tr>
</tbody>
</table>

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**Fig 1** *P*<O₂ (A) and *P*<CO₂ (B) in rats injected with endotoxin. All rats were mechanically ventilated at a peak inspiratory pressure of 25 cm H₂O and a PEEP of 7.5 cm H₂O for the indicated times. AS-30 (open triangles, n=8), AS-60 (open boxes, n=8) and AS-120 (open circles, n=8) groups underwent inhalation of an aerosolized MNS for 30, 60, and 120 min, respectively, from the starting point. The control group (closed circles, n=12) did not received aerosol. Pre-start: pre-starting period. Values show means (SD). *P*<0.05 vs the control group.
minutes after the starting point, the $P_{aO_2}$ of the AS-30, AS-60, and AS-120 groups had increased to 33.6 (16.0) kPa ($P<0.05$ vs control group). The $P_{aO_2}$ of the AS-30 and AS-60 groups, however, gradually decreased after the end of inhalation, and the values at 180 min after the starting point were not significantly different from that of the control group. In contrast, the value for the AS-120 group had increased to 46.1 (12.5) kPa at 120 min, and 52.1 (12.5) kPa 180 min after the starting point ($P<0.05$ vs control group).

$P_{aCO_2}$ of all rats was 3.5 (0.7) kPa during the baseline period, but increased to 7.3 (2.4) kPa in the pre-starting period with no significant inter-group differences. Changes of $P_{aCO_2}$ are shown in Figure 1A. The $P_{aCO_2}$ in the control group remained at approximately 7.3 kPa until the end of the experiment, and that in the AS-30 and AS-60 groups at approximately 6.7 kPa (N.S. vs control group). In contrast, the $P_{aCO_2}$ in the AS-120 group decreased gradually, reaching 5.3 kPa at 120 min after the starting point ($P<0.05$ vs control group).

Deflation limbs of the static pressure-volume recordings of the lung are shown in Fig. 2. Volumes of the AS-30 group did not differ significantly from those of the control group at any pressure. The volumes of the AS-60 group were significantly greater than those of the control group at airway pressures from 30 (maximal pressure) to 15 cm H$_2$O, while significant differences between the AS-120 and the control groups were observed at airway pressures from 30 to 5 cm H$_2$O.

Additional procedure
No peroxidase reactions were found in any lung sections stained without anti-SP-B antibody. A typical lung section of a rat with lung-lavage only stained with anti-SP-B is shown in Figure 3A, and that of one with lung-lavage plus inhalation of aerosolized MNS in Figure 3B. The presence of SP-B was detected immunohistochemically using anti-SP-B antibody (8B5E antibody), and was seen as brown granules resulting from peroxidase reaction. Magnification of the objective lens was ×20. Bars indicate 100 μm. (A) Brown granules were localized within some alveolar epithelial cells. Very little staining was found along the alveolar wall or terminal airways. (B) Parts of the alveolar wall and terminal bronchioles, as well as some alveolar epithelial cells, were stained with peroxide.

Discussion
In a randomized controlled clinical trial, it was reported that instillation of a MNS, Survanta, administered at 100 mg kg$^{-1}$ up to four times, improved short-term gas exchange and tended to improve survival rates in patients with
ARDS. The results of this trial seem to suggest that multiple administration at large doses of surfactant containing hydrophobic SPs is important. Bolus instillation of surfactant, however, caused a transient and significant decrease in arterial pressure in our previous study. Patients with ARDS or those at risk often manifest circulatory instability, so that bolus instillation may be difficult.

It was reported that slow continuous instillation of surfactant into the airway did not change arterial pressure in lung-lavaged rabbits. However, arterial blood gases were not improved because of inhomogeneous distribution of surfactant in the lung. We have shown that inhalation of aerosolized MNS improves blood gas exchange and lung mechanics without changing arterial pressure in rats with experimental ARDS caused by endotoxin. A recent study using a model of isolated perfused rabbit lung demonstrated that aerosolized surfactant improved ventilation/perfusion mismatch caused by inhalation of a detergent, Tween 20, and that a slow instillation of surfactant did not. The inhalation method, therefore, is superior to slow instillation.

Oxygenation index (\(P_{aO_2}, FIO_2\)) value for the criteria of ARDS is below 27 kPa and that of acute lung injury is below 40 kPa. In the present study, the oxygenation index of all the rats decreased to well below the criteria of ARDS (11.3 kPa) at the pre-starting point, as the result of the injection of the endotoxin. Inhalation of aerosolized MNS for 30 or 60 min initially restored gas exchange, but \(P_{aO_2}\) deteriorated after termination of the inhalation. In contrast, inhalation for 120 min caused an increase in \(P_{aO_2}\) to 46 kPa at the end of inhalation, and this increase continued after the end of the inhalation period. Inhalation for 120 min also improved \(P_{aCO_2}\) and static lung volume more than that for 30 or 60 min.

It is remarkable that the final \(P_{aO_2}\) value in AS-120 group was as high as the peak value for rats given 100 mg kg\(^{-1}\) by instillation of MNS into the airway in our previous study. In that study, intact rats were subjected to inhalation of MNS mixed with \(^{99m}\)TcO\(_4\). Only 4.2% of the total radioactivity was deposited in the lung. Based on this deposition rate, it was estimated that less than 64 mg kg\(^{-1}\) of MNS was deposited in the lungs of the AS-120 group rats as 600 mg was used for inhalation. Inhalation of aerosolized MNS was, therefore, more effective than bolus instillation in terms of the actual amount deposited.

In our additional procedure, deposition of the aerosolized MNS was assessed with the aid of anti-SP-B antibody. Location of aerosol deposition depends on the size of the aerosol. The diameter of the aerosolized MNS produced by our nebulizer (3.4 (1.2) \(\mu\)m) is close to the ideal size for alveolar deposition (2–3 \(\mu\)m). In the rats subjected to repeated lung-lavage without inhalation of aerosolized MNS, there was little staining along the airway or alveolar walls, indicating the effective removal of endogenous surfactant. In rats inhaling aerosolized MNS, the terminal airways and some alveolar walls were stained with peroxide, indicating the deposition of exogenous surfactant. A theoretical model for replacement therapy suggests that surfactant instilled into the airway moves to the alveoli when surface tension in the alveoli is elevated. We believe that aerosolized MNS deposited in both of the alveolar walls and the airways participated in reducing the alveolar surface tension in the main experiment.

In the present study, two of eight rats in the AS-120 group died because of hypotension, although \(P_{aO_2}\) values were maintained higher than 27 kPa. There were no significant differences in mortality rates among the four groups. Endotoxin causes hypotension and massive accumulation of ascites in rats, even when injected through the trachea. Treatment of hypotension may be important to assess the effects on mortality rate. It was reported that a modified bovine surfactant inhibited production of superoxide by neutrophils obtained from hamster with neutrophil alveolitis and promoted apoptosis of neutrophils in vitro. The effect of surfactant on neutrophils also needs further investigation.

We conclude that inhalation of aerosolized MNS can improve gas exchange and lung mechanics depending on duration of administration in the experimental setting used for this study. Inhalation for 2 h improved gas exchange to the same extent as was attained with bolus instillation. Prolonged inhalation of aerosolized surfactant, therefore, appears to be suitable for ARDS patients especially with circulatory instability.

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References


