Lung volumes and alveolar expansion pattern in immature rabbits treated with serum-diluted surfactant

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METHODS

Care of animals and preparation of test materials. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University and were approved by the Committee of the Ethics of Animal Experimentation of Kanazawa University, Takaratomy Campus. Serum was prepared from arterial blood collected from three adult rabbits anesthetized with intravenous pentobarbital sodium (10 mg/kg). Fibrin clots and cell components of the blood were removed by centrifugation, and the resulting sera were combined. The serum proteins were analyzed by electrophoresis (Climana-lizer JCA-MS24, Nihonkenshi, Tokyo, Japan) and the serum electrolytes by a conventional method (ABL 620, Radiometer, Copenhagen, Denmark).

Modified natural surfactant (MNS), consisting of 98% phospholipids (by weight), 0.9% other lipids, and 1.1% surfactant proteins B and C, was isolated with the aid of centrifugation and organic solvents from the alveolar lavage fluid of recently slaughtered pigs (12). The lypophilized MNS was then suspended in serum or acetate Ringer solution (R solution) at various concentrations (0.5–32 mg/ml).

Protocol 1 (ventilation with positive end-expiratory pressure). Thirty-eight immature rabbits were delivered at a gestational age of 26 days (term, 31 days) by hysterotomy from 9 pregnant does anesthetized with intravenous pentobarbital sodium (10 mg/kg) and local infiltration of 0.5% lidocaine (8 ml). They were anesthetized with intravenous pentobarbital sodium (0.5 mg), and an 18-gauge metal cannula was secured into the trachea through a tracheotomy. Then four to nine newborns from each litter were randomly assigned to one of two MNS groups or the reference group. The animals in the MNS groups were given 10 ml/kg of 2 mg/ml MNS in serum (2MNS-S group) or in R solution (2MNS-R group) via the tracheal cannula and before the first breath was taken. The animals in the reference group were further divided randomly into a serum-only group and a control group. The former received 10 ml/kg of serum only as described above, and no fluid was added to the lung of the latter.

IMMATURE NEWBORN RABBITS DELIVERED SEVERAL DAYS BEFORE TERM HAVE VERY LITTLE ENDOGENOUS SURFACTANT, AND THEIR LUNG FUNCTION DEPENDS ON THE QUALITY OF EXOGENOUS SURFACTANT INSTILLED INTO THE AIRWAYS (3, 11, 20). THESE ANIMALS MAY SERVE AS AN EXPERIMENTAL MODEL FOR TESTING THE HYPOTHESIS THAT ALVEOLAR OVERDISTENSION IS CAUSED BY DIssONATION AND INACTIVATION OF SURFACANT WITH THE PROTEINACEOUS EDema FLUID EVEN THOUGH THEIR PATHOLOGICAL FEATURES ARE DIFFERENT FROM ALI AND ARDS.

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delivering 100% oxygen at a frequency of 40 breaths/min. By measuring the pressure at the common tube of the ventilator circuit, we set the peak inspiration pressure at 22.5 cmH₂O and the peak end-expiratory pressure (PEEP) at 2.5 cmH₂O throughout the 20-min ventilation period. Tidal volumes were recorded every 5 min with a pneumotachograph system (12). Electrocardiograms were recorded after the period of ventilation, and animals with a heart rate of >100 beats/min were considered survivors.

The animals were then killed with an overdose of pentobarbital sodium, and the abdomen was opened to inspect the diaphragm for evidence of pneumothorax. They were left for 1 h at 37°C to induce lung collapse and connected in parallel to equipment for measuring the static pressure-volume (P-V) curve of the lung (12). Airway pressure was first raised stepwise from 0 to 30 cmH₂O and then lowered to 0 cmH₂O, with 1 min of equilibration at each 5-cmH₂O change. The volume measurements were corrected for gas compression in the equipment.

Next, a catheter was tied into the pulmonary trunk, after which the lungs were again inflated for 1 min at an airway pressure of 30 cmH₂O and then deflated to 10 cmH₂O. This pressure was maintained while the lungs were fixed by perfusion of the pulmonary artery for 60 min with 4% formalin. Large transverse sections of both lungs were obtained midway between the apex and base and subjected to image analysis to measure the size of the terminal air spaces. A microscopic image from the test grid enclosed by the lines of the test grid was sampled using a random start of one to six, according to the method recommended by Bolender et al. (4). By this method, two to four observational fields were sampled from each lung. The sizes of the alveolar and alveolar duct profiles within the sampled field were measured by the aid of a computer program (MicroAnalyzer, Nihon Poladigital, Tokyo, Japan). They were divided into five classes (a) <6 × 10⁴ µm², b) 6 to <20 × 10⁴ µm², c) 20 to <34 × 10⁴ µm², d) 34 to <48 × 10⁴ µm², and e) ≥48 × 10⁴ µm². For each of the animals, the percentage of each class to the whole was calculated.

Protocol 2 (ventilation without PEEP). Twenty-two immature rabbits were delivered from five does at a gestational age of 26 days. They were anesthetized and endotracheally cannulated in the same manner as described in protocol 1 and randomly divided into 24MNS-R, 2MNS-S, and control groups. Animals in the 24MNS-R group were given 10 ml/kg of 24 mg/ml MNS in R solution. No fluid was added to the lungs of the control group. PEEP was not used for these animals, but the other procedures were identical to those described in protocol 1. Waveform of the pressure at the common tube of the ventilator circuit was nearly rectangular, and the pressure at the end-expiratory period was 0 cmH₂O.

Measurement of dynamic surface tension. A MNS suspension (0.3–32 mg/ml in serum or in R solution) was placed in the sample chamber of a pulsating bubble system (Electronetics, Buffalo, NY), and a bubble was created in communication with the ambient air. After being heated to 37°C, the bubble was pulsed between radii of 0.40 and 0.55 mm at a speed of 40 cycles/min. The surface tension was calculated 3 min after the start of pulsation according to the law of Laplace (surface tension = pressure across the air-liquid interface × radius/2). The surface tension values at the minimum and maximum bubble sizes were designated as ymin and ymax, respectively. We also measured the values for serum only and those for 16 animals in the serum group and the whole was calculated.

Results

The sodium, potassium, calcium, and chloride concentrations in the serum were not very different from those in R solution (Na⁺ in serum/R solution, 138/130 mM; K⁺, 4.1/4.0 mM; Ca²⁺, 1.51/1.50 mM; Cl⁻, 101/109 mM). Proteins in the serum were mostly albumin (41 mg/ml) and globulin (13 mg/ml). No fibrinogen was identified in the serum.

In protocol 1, 13 animals were used in the 2MNS-S group, 12 in the 2MNS-R group, 7 in the serum-only group, and 7 in the control group. Two animals in the 2MNS-S group developed pneumothorax and were excluded from this study. Two animals in the serum-only group and one animal in the control group died during the ventilation period, but we did not exclude them from this study. The pneumothorax and survival rates were not significantly different among the groups. The mean body weight of all the animals included in the statistical analysis (n = 37) was 25.9 ± 4.5 g, and there were no significant intergroup differences.

In protocol 2, eight animals were used in the 24MNS-R group, seven in the 2MNS-S group, and seven in the control group. No pneumothorax was observed. One animal in the 2MNS-S group and two animals in the control group died during the ventilation period, but all of these animals were included in this study. No significant differences in survival rates were seen among the groups. The mean body weight of all the animals (n = 22) was 26.8 ± 4.2 g without significant intergroup differences.

Figure 1A shows the tidal volumes measured in protocol 1. The control group had no more than 3 ml/kg of tidal volume. The data for the serum-only group were omitted from the figure, because they were nearly the same as those for the control group. In the 2MNS-R group, one animal (1 of 12) had some tidal volume (9.0 ml/kg) at the end of the ventilation period, but the mean value (3.9 ± 4.7 ml/kg) was not significantly different from the control group. In contrast, the volume of the 2MNS-S group increased with the duration of ventilation (P < 0.05), registering 7.2 ± 5.8 ml/kg at 5 min and 14.7 ± 7.4 ml/kg at 20 min after the start of ventilation (P < 0.05 vs. other groups).

Figure 1B shows the tidal volumes observed in protocol 2. The control group had no more than 3 ml/kg of tidal volume. The data for the 2MNS-S group were not significantly different from those for the control group in the absence of PEEP. In contrast, the 24MNS-R group had a tidal volume of 7.8 ± 5.6 ml/kg at 5 min and 15.1 ± 8.1 ml/kg at 20 min after the start of ventilation (P < 0.05 vs. other groups). The tidal volume of this group increased with the duration of ventilation (P < 0.05).

Figure 2A shows the static P-V curves of the lung-thorax system measured in protocol 1. Static lung volumes of the control group and the serum-only group (data omitted in the figure) were <10 ml/kg at any airway pressure. The volumes of the 2MNS-R group were not significantly different from those of the control group. On the other hand, the 2MNS-S group had a static lung volume of 56.3 ± 17.8 ml/kg at an airway pressure of 30 cmH₂O. At airway pressures of 25 cmH₂O during inflation and from 30 to 5 cmH₂O during deflation, the 2MNS-S group had significantly larger lung volumes than the other groups in this protocol (P < 0.05).
during deflation, the 24MNS-R group had significantly smaller tidal volumes than the other groups in this protocol ($P < 0.05$). Values for the 2MNS-S group for protocol 1 were not significantly different from those for the control group, whereas the 24MNS-R group had a volume of 64.8 ± 3.4 ml/kg at any airway pressure of 30 cmH$_2$O. At airway pressures of 20 and 25 cmH$_2$O during inflation and from 30 to 5 cmH$_2$O during deflation, the 24MNS-R group had significantly larger volumes than the other groups in this protocol ($P < 0.05$).

Tidal volumes and static lung volumes for the 2MNS-S group in protocol 1 were not significantly different from those for the 24MNS-R group in protocol 2. In contrast, the volume data for the animals in the 2MNS-S group were significantly different between protocol 1 (with PEEP) and protocol 2 (without PEEP).

Figure 3 shows representative lung sections (top) and histograms for the size class distribution of the alveolar and alveolar duct profiles (bottom). These images correspond to a specific point on the deflation limb of the P-V curve: 10 cmH$_2$O (see Fig. 2). In the control group for protocol 1, all alveoli and alveolar ducts were in a state of collapse, and the smallest class accounted for 79 ± 8% of the total open spaces. In the 2MNS-S group for protocol 1, the largest class accounted for 38 ± 8% of the total open spaces (highest of all groups, $P < 0.05$), and the smallest class accounted for 31 ± 9% (lower than control group, $P < 0.05$; higher than 24MNS-R group, $P < 0.05$). Moreover, the alveoli and alveolar ducts of the largest and the smallest classes were frequently located next to each other. In the 24MNS-R group of protocol 2, almost all of the alveoli and alveolar ducts were well aerated, with the smallest class accounting for 16 ± 6% (lowest of all groups, $P < 0.05$). Findings of the lung sections not presented here (serum-only and 2MNS-R groups for protocol 1, and control and 2MNS-S groups for protocol 2) were not significantly different from those of the control group for protocol 1.

Figure 4 shows the dynamic surface tension of MNS in the serum and R solution at various concentrations. The yin
values for both MNS in R solution and in serum were <3 mN/m at MNS concentrations of 16 mg/ml and higher. The value for the former remained at a low level until the MNS concentration decreased to 2 mg/ml, but that for the latter rose to over 20 mN/m when the concentration decreased to 8 mg/ml ($P < 0.05$). At an MNS concentration of 0.5 mg/ml, the mean $\gamma_{\text{min}}$ values were 23 mN/m for MNS in R solution and 34 mN/m for MNS in serum ($P < 0.05$).

The $\gamma_{\text{max}}$ values for both MNS in R solution and in serum were $\sim$30 mN/m at MNS concentrations of 16 mg/ml and higher. The value for MNS in R solution remained at the same level until the concentration decreased to 4 mg/ml, but that for MNS in serum rose to 44 mN/m when the concentration decreased to 8 mg/ml ($P < 0.05$). However, when the concentration decreased to 0.5 mg/ml, the $\gamma_{\text{max}}$ value for the former rose to 69 mN/m, whereas that for the latter remained below 50 mN/m ($P < 0.05$).

The test material of serum only had a $\gamma_{\text{min}}$ of 39 ± 2 mN/m and a $\gamma_{\text{max}}$ of 54 ± 2 mN/m ($n = 6$). With Tween 20 at a concentration of 30 mg/ml, 16 mg/ml MNS in R solution had a $\gamma_{\text{min}}$ of 24 ± 1 mN/m and a $\gamma_{\text{max}}$ of 31 ± 1 mN/m ($n = 6$; difference between $\gamma_{\text{min}}$ and $\gamma_{\text{max}} = \sim 7$ mN/m).

**DISCUSSION**

The 2MNS-S group for protocol 1 (given serum-diluted MNS and ventilated with PEEP) had nearly the same tidal volume and static P-V curve as the 24MNS-R group of protocol 2 (given nondiluted MNS and ventilated without PEEP) but showed different morphometric findings in the lung sections. The former group had significantly higher percentages of alveoli and alveolar ducts in the largest and the smallest classes than the latter group, indicating that the majority of alveoli and alveolar ducts of the former were either overdistended or collapsed.

It has been reported in animals with experimental ARDS that proteinaceous edema fluid similar to plasma appears in the alveoli and that the concentration of surfactant lipid decreases.
to below 1 mg/ml (16). The immature rabbits used in our study have very little endogenous surfactant (12, 20), because the control group showed no more than 3 ml/kg of tidal volume. The alveoli of neonates are filled with ~30 ml/kg of fetal lung liquid before the first breath is drawn (26). Animals in the 2MNS-S group were given 10 ml/kg of 2 mg/ml MNS suspended in serum. If we assume that the administered fluid was evenly mixed with the fetal lung liquid, the concentration of MNS in the alveoli would become ~0.5 mg/ml. Therefore, the concentration of surfactant at the alveoli of the 2MNS-S group would resemble that seen in ARDS.

The law of Laplace indicates that the larger alveoli would become larger and the smaller alveoli smaller at a certain airway pressure, if the surface tension did not alter during cyclic change of the surface area. Tween 20, which causes airway pressure, if the surface tension did not alter during recruitment of some alveoli was also observed when PEEP was applied in the present animals in the 2MNS-S group. It may therefore be necessary to examine the pattern of alveolar distension or recruitment by elevating the PEEP. In our preliminary study, however, elevation of PEEP considerably increased the incidence of pneumothorax in animals receiving the serum-diluted MNS (data not shown). Even with the PEEP level used in the present study, 2 of the 13 animals developed pneumothorax. Other ventilator settings (higher PEEP, lower peak inspiration pressure) might improve the alveolar expansion pattern and reduce the incidence of pneumothorax. In addition, the immaturity of the lungs cannot be excluded as a factor of the pneumothorax. We suppose, however, that the pneumothorax may have been induced by alveolar overdistension, which developed after the recruitment.

Studies with computerized tomography suggest that the recruitment of alveoli occurs progressively from nondependent to dependent lung regions in ARDS (6, 17), but our study showed that the recruitment differs even between neighboring alveoli. In addition, it is reported that alveoli injured by Tween 20 are recruited throughout the generation of the P-V curve (22). These findings suggest that the recruitment of alveoli with inactivated surfactant is very complex and needs further study.

It has been reported that mechanical ventilation with a lower tidal volume than is traditionally used results in a reduced mortality of ALI and ARDS patients, presumably because alveolar overdistension is prevented (1, 9). Some controversy, however, still surrounds this technique (5). In our study, serum lost its effect on the surface properties of MNS, when the concentration of MNS was increased to 16 mg/ml and higher. This result suggests that surfactant replacement could be a rational means for the prevention of alveolar overdistension in ALI and ARDS patients if a sufficient dose of effective surfactant could be supplied to the alveoli.

In protocol 1, the 2MNS-S group had a larger tidal volume and also a larger static lung volume than the 2MNS-R group. This phenomenon seems to be inconsistent with the notion that proteins in serum inhibit pulmonary surfactant activity (10, 11, 13, 16). However, when the MNS concentration was reduced to 0.5 mg/ml, the ymax value of MNS in serum was ~19 mN/m lower than that in R solution. The lower ymax can be expected to reduce the alveolar contractile force at end inspiration, thus improving lung mechanics. This speculation needs to be verified.

The tidal volume of some groups increased with the duration of ventilation. A similar phenomenon has been seen in immature lambs receiving exogenous surfactant (3). This increase may reflect gradual refinement of the alveolar surface film and postnatal absorption of lung liquid. MNS lacks surfactant protein A, which plays a role in the activity of pulmonary surfactant (5a, 27), and serum lacks fibrinogen, which is a potent surfactant inhibitor (15, 24). The influence of these substances on the function of diluted surfactant needs to be examined. In addition, longer duration for the mechanical ventilation than was used in our study may be required to mimic more closely the clinical situation in ALI and ARDS.

Various questions remain to be addressed. We conclude, however, that administration of serum-diluted surfactant to immature neonatal lungs leads to irregular alveolar expansion, with patchy overdistension of terminal air spaces, similar to the expansion pattern that may be seen after dilution of endogenous surfactant with proteinaceous edema fluid in ALI and ARDS.

**Fig. 4.** Minimum surface tension (circles) and maximum surface tension (triangles) of MNS suspended in acetate Ringer solution (open symbols) and in serum (closed symbols) at various concentrations. Values are means ± SD of n = 5–7 measurements. *P < 0.05 vs. corresponding concentrations of MNS in acetate Ringer solution.
REFERENCES


