Application of a new ventilator-multi-plethysmograph system for testing efficacy of surfactant replacement in newborn rabbits

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ABSTRACT: We applied a new ventilator-multi-plethysmograph system to evaluate the effect of surfactant replacement in newborn rabbits under well controlled, nearly physiological conditions characterized by normal ECG and adequate Pco2, in right ventricular heart blood obtained at the end of the experiment. Up to 10 animals were ventilated in parallel with a pressure-constant common respirator system. Using a working pressure of 4.9 kPa (50 cmH2O), we could adjust the pressure delivered to each animal within the range of 0.49-4.4 kPa (5-45 cmH2O), by changing the length of an open high-resistance tube constituting the outflow limb of the connection between the common ventilator tube and the tracheal cannula. Immature newborn animals obtained after 27.5 days gestation and ventilated for 30 min with a tidal volume of 8-10 ml/kg had a mean lung-thorax compliance of 4.2±1.1 ml·kPa^{-1}·kg^{-1} (0.41±0.11 ml·cmH2O^{-1}·kg^{-1}) and Pco2 of 8.5±1.9 kPa. In littersmates treated at birth with a large dose of natural surfactant (Curosurf, 200 mg·kg^{-1}), compliance increased to 6.0±1.0 ml·kPa^{-1}·kg^{-1} (0.68±0.10 ml·cmH2O^{-1}·kg^{-1}) (p<0.01) and Pco2 decreased to 6.9±1.2 kPa (p<0.01). Near-term animals, obtained at 30 days gestation and ventilated under similar conditions had a compliance of 7.2±0.9 ml·kPa^{-1}·kg^{-1} (0.71±0.09 ml·cmH2O^{-1}·kg^{-1}) and Pco2 of 6.4 (1.2) kPa. Administration of surfactant (same dose as above) to these mature animals at birth had no adverse effects.


Surfactant deficiency is a main aetiologic factor in the pathogenesis of the neonatal respiratory distress syndrome (RDS), as underscored by data from clinical trials showing efficacy of surfactant replacement therapy even in severe forms of this disease [1, 2]. The surfactants used in these and other [3, 4] clinical trials were first tested extensively for biophysical activity under various experimental conditions including instillation of the material into the airways of artificially-ventilated preterm newborn animals [5-8]. Rabbit foetuses, delivered prematurely at a gestational age of 27 days (term 31 days) provide a well-established experimental model for this purpose [9, 10]. With the body-plethysmograph system designed by LACHMANN et al. [11], multiple newborn rabbits can be subjected in parallel to pressure-constant ventilation, while the response to surfactant replacement therapy is evaluated by tidal volume measurements [12]. In the original version of this device, the insufflation pressure was not adjusted to the individual requirements and physiological conditions were, therefore, usually not attained. Although in a population of animals average tidal volumes might seem adequate at a certain ventilator setting, individual animals were frequently either overventilated or underventilated. Using an intricate construction of values and waterlocks, IKUGAMI et al. [13] recently developed an alternative body-plethysmograph system for multiple animals, allowing individual adjustment of the pressure generated from a common ventilator source. In the present paper, we describe a more convenient solution to the same problem, originally designed by one of us (T. Kobayashi, unpublished). We have applied this innovation to studies on immature newborn rabbits in order to delineate new experimental criteria for a satisfactory therapeutic response to surfactant replacement therapy.

Materials and methods

The ventilator-multi-plethysmograph system

The design of this modified ventilator-multi-plethysmograph system is illustrated in figure 1. From a common respirator unit (Servo Ventilator 900 B, Siemens- Elema, Solna, Sweden), a large gas flow
(5 l/min) is delivered under constant pressure during the inspiratory phase of each ventilatory cycle. This gas (100% oxygen in the present experiments) flows through a wide tube (cross-sectional area: 1 cm²), to which the tracheal cannula of each animal is connected by means of a narrow, high-resistance silicon tube (I.D. 1 mm) with an open outflow limb. The pressure drop from the large common ventila-tor tube to the end of each narrow tube is determined by the length of this latter tube, and the pressure delivered to the experimental animal can thus be reduced simply by cutting the outflow tube, and increased by adding pieces of tubing on the same side. During these maneuvers, the inflow side of the tube is clamped to avoid sudden high peaks of inflation pressure due to accidental obstruction of the outflow tube. Pressure can also be modified by altering the length of the inflow limb. At a point opposite to the connection between the high-resistance silicon tube and the tracheal cannula, the pressure delivered to each animal was measured by means of a conventional pressure transducer (EMT 34, Siemens-Elema). The connections between the inflow and outflow portions of the narrow tube, the tracheal tube, and the exit to the pressure transducer were enclosed in a plastic block, attached directly to the plethysmograph wall to minimize deadspace.

With this arrangement and with the ventilator set at a working pressure of 4.9 kPa (50 cmH₂O) we could ventilate 10 animals in parallel, adjusting the individual insufflation pressure within the range of 0.49-4.4 kPa (5-45 cmH₂O) (fig. 2). Tidal volumes were measured by means of a specially designed "Fleisch-tube" [14] connected to the other side of the plethysmograph box, a differential pressure transducer (EMT 32, Siemens-Elema), an amplifier (EMT 31, Siemens-Elema), an integrator unit (EMT 41, Siemens-Elema) and a recorder (Mingograf 81, Siemens-Elema).

Throughout the present experiments, the ventilator was set at a frequency of 40 cycles·min⁻¹ and 50% inspiration time. No positive end-expiratory pressure was applied. The ECG was recorded at regular intervals from subcutaneous needle electrodes connected to the animal inside the plethysmograph box.

![ECG diagram](image)

**Fig. 1.** The design of the ventilator-multi-plethysmograph system applied in the present experiments. From the common ventilator tube, narrow high-resistance tubes lead to each tracheal cannula, continuing as open outflow limbs. Tidal volumes are measured with a "Fleisch-tube" connected to the opposite side of the plethysmograph box. The pressure delivered to each animal is kept at the level required to maintain the standardized tidal volume by adjusting the length of the open outflow limb of the high-resistance tube. *: flow and volume; Δ: pressure transducer.

**Fig. 2.** Recordings documenting individual variations in insufflation pressure, ranging from 0.49-4.4 kPa (5-45 cmH₂O) while the working pressure of the common ventilator system is set at 4.9 kPa (50 cmH₂O).

**Preparation and characterization of surfactant**

Surfactant ("Curosurf") was isolated from minced pig lungs by a combination of washing, centrifugation, extraction with chloroform:methanol and liquid-gel chromatography, as described previously [7, 15]. Containing approximately 99% polar lipids, mainly phospholipids, and 1% hydrophobic proteins (SP-B and SP-C) [15], this surfactant lowers the contractile force at an air-liquid interface to near 0 nM·m⁻¹ during surface compression [15]. The physiologic activity of the batches used in the present experiments had been confirmed in earlier experiments on premature newborn rabbits ventilated with a standardized sequence of insufflation pressures (data not shown) as well as in clinical trials of surfactant replacement therapy for neonatal RDS [2]. Surfactant was administered at the same concentration as in the clinical trials, 80 mg·ml⁻¹.

**Protocols for animal experiments**

Newborn rabbits obtained by hysterotomy at a defined gestational age (see below) were anaesthetized at birth by intraperitoneal injection of 0.1 ml sodium pentobarbital (Mebumal vet. 6 mg·ml⁻¹, Nord Vacc, Skärholmen, Sweden) and tracheotomized. They were randomized either to receive surfactant suspension via the metal tracheal cannula or to serve as controls in which no material was instilled into the airways. After the tracheotomy and instillation procedures (average duration about 3 min), the animals were relaxed by intraperitoneal injection of 0.1 ml pancuronium
bromide (Pavulon, 0.2 mg·ml\(^{-1}\), Organon, Oss, Holland) and kept at 37°C in the plethysmograph system, connected to the respirator unit. ECG electrodes were inserted before the box was closed, and the animal was not included in the study unless regular cardiac activity with normal QRS-complexes was recorded at the onset of ventilation. The tidal volumes were adjusted as described above, and a stable level could usually be attained within approximately 5 min after the onset of ventilation. For the rest of the experiments, two protocols were applied.

High vs low tidal volumes in immature animals (Protocol 1). These experiments were designed to investigate whether gas exchange and survival would differ between animals ventilated with small or large tidal volumes. A total of 50 immature newborn rabbits were obtained from 6 does at a gestational age of 27 days. These animals received either 0.1 ml surfactant [16] or served as controls, and in each of these groups the tidal volumes were adjusted to either 6-8 ml·kg\(^{-1}\) or 10-12 ml·kg\(^{-1}\). The animals were ventilated for 60 min, and simultaneous recordings of tidal volume, pressure, and ECG were obtained every 15 min. Compliance values for the respiratory system (lung-thorax compliance) were calculated from the quotient between tidal volume and peak insufflation pressure.

Final protocol, applied for testing multiple surfactant batches (Protocol 2). A total of 108 immature newborn rabbits were obtained from 16 does at a gestational age of 27.5 days. They were tracheotomized and received the same dose of surfactant as used in our clinical trials, 200 mg·kg\(^{-1}\) body weight [2], or served as non-treated controls. Seven batches of surfactant were examined, 1-2 batches in each experiment. This implies that data for each batch were obtained from 2-6 litters (median = 3). The animals were ventilated for 30 min with an intermediate tidal volume (8-10 ml·kg\(^{-1}\)), adjusted as described above. (The reasons for these modifications of protocol 1 are given below).

To investigate whether the exogenous surfactant would have any adverse effects in mature animals, we also included 13 near-term rabbits obtained from 2 does at a gestational age of 30 days. These animals were ventilated under the same conditions as in the final protocol, with or without previous treatment with surfactant.

After the period of ventilation, the animals were killed by intracranial injection of 0.5 ml Lidocaine (Xylocaine, 20 mg·ml\(^{-1}\), Astra, Södertälje, Sweden) (this leads to immediate cardiac arrest). The abdomen was opened and the diaphragm inspected for pneumothorax; thereafter the chest was opened in the anterior midline and blood was drawn from the right ventricle for determination of carbon dioxide tension (P\(_{CO_2}\)).

In animals with evidence of pneumothorax, the moment of lung rupture could usually be identified retrospectively in our tracings from a sudden drop of tidal volume by more than 50%, occurring during ventilation with the same insufflation pressure [16].

Preparation and histological examination of lungs

The lungs were fixed by vascular perfusion with a mixture of 3.5% formaldehyde and 1% glutaraldehyde, while kept expanded at a deflation pressure of 0.98 kPa (10 cm H\(_2\)O) [10]. They were embedded in paraffin and transverse sections from the lower lobes (stained with haematoxylin and eosin) were examined with particular reference to alveolar expansion pattern and epithelial necrosis in conducting airways [17]. The volume density of the alveolar spaces was determined by point-counting, using the total parenchyma as a reference volume [10].

Statistical methods

Differences between the groups were evaluated with the Wilcoxon Mann-Whitney two-sample test (two-tailed) and with the Chi-squared test, using p=0.05 as the limit level of statistical significance.

Results

Physiological measurements from Protocol 1

The overall survival rate was 44% in this series of experiments, with no difference between surfactant-treated animals and controls or between animals ventilated with the lower or higher tidal volume range (table 1). Pneumothorax was a frequent complication, with a total incidence of no less than 50%. The majority of the pneumothorax episodes (17/25) occurred during the second 30 min of ventilation. The incidence of
The results are given as mean±sd. All animals were delivered at a gestational age of 27 days and ventilation for 60 min. **: p vs control group with same tidal volume range <0.01. Pco₂: carbon dioxide tension.

Pneumothorax did not differ significantly between surfactant-treated animals and controls or between animals ventilated with lower or higher tidal volumes. Among surfactant-treated animals without pneumothorax, two in each group were excluded because of ECG abnormalities appearing during the period of ventilation. Physiological data from experiments testing 7 batches of surfactant are shown in figure 4. Average tidal volumes were generally close to the upper limit of the standardized range, and in one group of surfactant-treated animals were slightly above 10 ml·kg⁻¹. The mean compliance improvement in surfactant-treated animals, reflected by the significantly lower pressure requirements, amounted to 6.9%: 6.9 vs 4.2 ml·kPa⁻¹·kg⁻¹ (0.68 vs 0.41 ml·cmH₂O⁻¹·kg⁻¹) (p<0.001). Average Pco₂ was significantly lower in surfactant-treated animals than in controls: 6.9 vs 8.5 kPa (p<0.001).

Corresponding physiological data from near-term newborn rabbits are included in table 3 and figure 4. There were no significant differences in insufflation pressure, compliance or Pco₂ between surfactant-treated and non-treated mature animals. The values for compliance and Pco₂ in these animals were 7.2±0.9 ml·kPa⁻¹·kg⁻¹ (0.71±0.09 ml·cmH₂O⁻¹·kg⁻¹), and 6.4±1.2 kPa, respectively.

Histological and morphometric observations

The lungs of surfactant-treated immature animals (both protocols) had a nearly uniform alveolar expansion pattern with well aerated terminal airspaces and usually well preserved epithelium in conducting airways. In littermate

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Table 2. — Physiological data from animals studied according to Protocol 1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tidal volume ml·kg⁻¹</th>
<th>Insufflation pressure kPa</th>
<th>Lung-thorax compliance ml·kPa⁻¹·kg⁻¹</th>
<th>Pco₂ kPa</th>
<th>Heart rate per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>6.6±0.8</td>
<td>2.55±0.20</td>
<td>2.7±0.3</td>
<td>7.8±3.6</td>
<td>285±28</td>
</tr>
<tr>
<td>Surfactant</td>
<td>5</td>
<td>7.0±1.1</td>
<td>1.67±0.49**</td>
<td>4.3±1.0**</td>
<td>6.5±3.7</td>
<td>323±17</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>10.7±0.5</td>
<td>2.36±0.59</td>
<td>4.9±1.4</td>
<td>6.8±1.1</td>
<td>317±12</td>
</tr>
<tr>
<td>Surfactant</td>
<td>5</td>
<td>11.5±0.3</td>
<td>1.86±0.49</td>
<td>6.6±1.5</td>
<td>5.6±2.1</td>
<td>306±9</td>
</tr>
</tbody>
</table>

The lungs of surfactant-treated immature animals (both protocols) had a nearly uniform alveolar expansion pattern with well aerated terminal airspaces and usually well preserved epithelium in conducting airways. In littermate
controls, on the other hand, alveolar air expansion was characteristically incomplete and irregular, and necrosis of airway epithelium was a common finding. The difference between surfactant-treated animals and controls was further corroborated by our morphometric findings (tables 4 and 5): the alveolar volume density was, on average, increased by 61% in immature animals receiving surfactant (table 5). Treatment with surfactant had no effect on the alveolar expansion pattern of near-term animals (table 5).

### Discussion

The data from the present experiments provide some new guidelines for experimental evaluation of surfactant substitutes in artificially ventilated immature newborn rabbits. Contrary to earlier experiments [18] involving tidal volume measurements in animals ventilated with a standardized sequence of different insufflation pressures, our present methods were designed to provide adequate lung perfusion and gas exchange during the period of ventilation, as documented by normal ECG and...
acceptable final values of $P_{\text{aco}}$ in heart blood. In our opinion, the equipment described in this paper is more convenient to use than the elaborate system of valves and adjustable water-locks designed for the same purpose by Ikemami et al. [13]. We also believe that our modified ventilator-multi-plethysmograph system may be applicable to other experimental models of neonatal lung disease, based on long-term ventilation of more mature animals.

In our experiments on animals delivered according to Protocol 1 (tables 1 and 2), pneumothorax was an acceptably frequent complication irrespective of whether the animals were ventilated with a low (6-8 ml·kg$^{-1}$) or a high (10-12 ml·kg$^{-1}$) tidal volume. The tendency to lung rupture was probably related to structural factors including the low content of elastin and collagen in very immature lungs [19]. Although the rupture was no doubt triggered by the ventilatory pressure waves, the incidence of pneumothorax was not directly related to the pressure required to maintain the standardized tidal volume. Pneumothorax was as common in surfactant-treated animals as among controls, although the former animals could be ventilated with a significantly lower pressure. Our “retrospective” analysis of the tidal volume trajectories from animals delivered after 27 days of gestation furthermore revealed that the episodes of pneumothorax mainly occurred during the second 30 min of ventilation.

These observations prompted us to modify our first protocol by delivering the animals approximately 12 h later (corresponding to 27.5 days of gestation) and shortening the period of ventilation to 30 min. We also chose to ventilate the animals with an intermediate tidal volume (8-10 ml·kg$^{-1}$) similar to that needed for healthy preterm newborn babies [20]. With this new protocol, pneumothorax was a much less frequent complication (table 3), and the animals could usually be maintained in stable conditions during the scheduled period of ventilation, with acceptable $P_{\text{aco}}$ at the end of the experiment.

In principle, the response to surfactant replacement therapy varies with the degree of lung maturity in the treated animals [10, 21]. In an extremely immature foetal rabbit lung without fully developed terminal air sacs (<26 days of gestation), compliance levels close to those of a full-term lung cannot be reached simply by upgrading surfactant-deficient lungs which have just entered the terminal-sac stage of development respond very well to surfactant replacement therapy. However, the animals amount to 95% of the animals receiving surfactant at birth show no further increase in compliance (table 3), and the animals could usually be maintained in stable conditions during the scheduled period of ventilation, with acceptable $P_{\text{aco}}$ at the end of the experiment.

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