Biophysical and physiological properties of a modified porcine surfactant enriched with surfactant protein A

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Biophysical and physiological properties of a modified porcine surfactant enriched with surfactant protein A. B. Sun, T. Curstedt, G. Lindgren, B. Franzén, A.A. Alaiya, A. Čalkovská, B. Robertson. ©ERS Journals Ltd 1997.

ABSTRACT: Surfactant protein A (SP-A), a major protein component of natural pulmonary surfactant, is absent in exogenous surfactants currently used in clinical practice.

We investigated the physical and physiological properties of one of these modified natural surfactants (Curosurf®) after enrichment with 5% SP-A (SP-A-Curosurf). A pulsating bubble system was used for *in vitro* assessments and ventilated newborn rabbits for evaluation of *in vivo* effects.

In the presence of various potential inhibitors (meconium 5 mg·mL⁻¹, fibrinogen 5 mg·mL⁻¹, albumin 25 mg·mL⁻¹, or whole serum proteins 25 mg·mL⁻¹), Curosurf at a concentration of 5 mg·mL⁻¹ was inactivated while SP-A-Curosurf and natural porcine surfactant at the same concentration had normal maximum and minimum surface tension. This protective effect of SP-A was calcium dependent. In immature newborn rabbits, the improvement of lung-thorax compliance observed after treatment with 100 mg·kg⁻¹ of SP-A-Curosurf was equivalent to that obtained with 200 mg·kg⁻¹ of Curosurf. Similarly, in near-term newborn rabbits with respiratory failure induced by instillation of fibrinogen *via* the airways, the increase in compliance after administration of 100 mg·kg⁻¹ of SP-A-Curosurf corresponded to that seen after treatment with 200 mg·kg⁻¹ of Curosurf, whereas Curosurf at a dose of 100 mg·kg⁻¹ had no substantial effect.

Our data thus indicate that surfactant protein A increases the resistance of Curosurf to inactivation under *in vivo* conditions.

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Lung immaturity and surfactant deficiency are the main factors in the pathogenesis of neonatal respiratory distress syndrome (RDS). Surfactant dysfunction, caused by inactivation of surface active material in the airspaces, also contributes to respiratory failure in other forms of neonatal lung disease, such as meconium aspiration syndrome (MAS), congenital pneumonia, congenital diaphragmatic hernia, and in the adult respiratory distress syndrome (ARDS) [1].

Pulmonary surfactant is a mixture of phospholipids and specific apoproteins produced by alveolar type II cells [2]. It reduces surface tension of the alveolar lining layer, facilitates foetal lung liquid absorption at birth, and prevents transudation of oedema fluid into the alveolar spaces. In modified natural surfactant substitutes isolated by extraction with organic solvents, the major surface active components are saturated phospholipids, mainly dipalmitoylphosphatidylcholine (DPPC), and the hydrophobic surfactant proteins B and C (SP-B, SP-C). In complete natural surfactant, surfactant protein A (SP-A) is the major protein constituent. Its native form is an aggregated oligomer of 18 glycoprotein peptides, each with a molecular size of 30–35 kDa. It regulates surfactant phospholipid metabolism, facilitating recycling of endogenous surfactant between the

type II cells and the alveolar compartment [3, 4], and regulates transformation of lamellar bodies to tubular myelin [5]. Several investigators have reported that the presence of SP-A makes surfactant more resistant to inactivation by blood proteins *in vitro* [6, 7] as well as in ventilated newborn experimental animals [8]. YAMADA *et al.* [9] found that addition of SP-A to modified bovine surfactant (Survanta®, Abbott, North Chicago, IL, USA) potentiated the therapeutic effect of the surfactant on dynamic and static lung compliance in ventilated premature newborn rabbits.

Curosurf® (Chiesi Farmaceutici, Parma, Italy) is another modified natural surfactant, widely used in clinical practice. This surfactant, isolated from pig lungs by extraction with organic solvents and liquid-gel chromatography (see below), contains only polar lipids, SP-B and SP-C. Excellent results have been reported from randomized clinical trials [10], but the recommended dose of Curosurf for prevention and treatment of RDS is comparatively high (200 mg·kg⁻¹). In the present paper, we investigate whether the physical and physiological properties of Curosurf, including its dose-effectiveness and resistance to inactivation by meconium and plasma proteins, can be improved by enrichment with SP-A.

Materials and methods

Preparation of surfactants and SP-A

Isolation of natural surfactant. Natural porcine surfactant (NPS) was prepared from saline (0.9% NaCl) lavage of fresh pig lungs as reported elsewhere [9], with minor modifications. Briefly, the lavage fluid was centrifuged at $5,000 \times g$ and 4°C for 2 h. The supernatant was removed, and the sediment diluted with saline and centrifuged again over a 0.68 M sucrose gradient at $5,000 \times g$ and 4°C for 30 min. The surfactant pellicle at the sucrose-saline interface was collected, suspended in saline, and centrifuged at $15,000 \times g$ and 4°C for 30 min. The latter procedure was repeated twice and the resulting white and creamy surfactant pellet was diluted with 0.45% NaCl. Its phospholipid content was determined according to BARTLETT [11], and the suspension was thereafter readjusted to a total phospholipid concentration of $40 \text{ mg}\cdot\text{mL}^{-1}$ by partial lyophilization. The final product was stored at -20°C . DPPC accounts for approximately 45% of the phosphatidylcholine (PC) fraction in this surfactant as determined by gas-liquid chromatography [12]. The NPS preparation was used for both *in vitro* and *in vivo* experiments (see below), as well as for isolation of SP-A.

Isolation of SP-A. The surfactant was added in droplets to stirred 1-butanol (Merck, Darmstadt, Germany; 2 mL butanol per mg surfactant phospholipid), and the stirring continued for 60 min at room temperature, followed by centrifugation at $1,000 \times g$ and 4°C for 30 min. The sediment was suspended in butanol and centrifuged twice again under the same conditions, then thoroughly dried with nitrogen and resuspended in 2–3 mL of distilled water. This suspension was centrifuged at $1,000 \times g$ and 20°C for 15 min, and approximately 2 mL of the supernatant was recovered and loaded on a 45 cm Sephadex G-200 column (Pharmacia, Uppsala, Sweden; bed volume 50 mL) and eluted with 40 mL of distilled water at a rate of $2\text{--}3 \text{ mL}\cdot\text{h}^{-1}$. This procedure was carried out at 4°C . Collected fractions were immediately frozen and lyophilized, and the dry weight of the protein was determined with an analytical balance (Mettler-Toledo AE-163, Greifensee, Switzerland) at a precision of 0.01 mg. Aliquots from each fraction were dissolved in phosphate buffered saline (PBS; 50 mM phosphate buffer in 150 mM NaCl), pH 7.40, at a concentration of $5 \text{ mg dry weight}\cdot\text{mL}^{-1}$, and the protein content was checked by high performance liquid chromatography (HPLC). The protein samples were loaded on a cross-linked agarose prepac column (NovaroseTM SE-1000/17, $8 \times 300 \text{ mm}$, volume 15 mL; Inovata AB, Bromma, Sweden), which separates proteins according to their molecular size. Exclusion limit for globular proteins for the column is approximately 1,000 kDa. The column was connected to an HPLC pump (Pharmacia-LKB 2248, Bromma, Sweden) and eluted at room temperature with 50 mM PBS, pH 7.40, as mobile phase, at a flow rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$ with a system pressure of 300 kPa. Eluates were monitored at 280 nm by a detector (Pharmacia-LKB 2510 Uvicord, Bromma, Sweden). Twenty to 100 μL was loaded for each run. Molecular size and isoforms of SP-A were identified with a standard two-dimensional isoelectric-focusing polyacrylamide

gel electrophoresis method with minor modification as reported elsewhere [13].

Analysis of SP-A with two-dimensional gel electrophoresis (2-DE). Detailed descriptions of the 2-DE technique have been published elsewhere [14, 15]. Briefly, purified SP-A was solubilized in a solution containing a high concentration of urea, dithiothreitol (DTT), non- and zwitter-ionic detergents and Resolyte 4–8 (BDH Laboratory Supplies, Poole, England). Approximately 1 μg protein was subjected to isoelectric focusing in $1.2 \times 180 \text{ mm}$ rod gels containing Resolyte 4–8. After short equilibration in a sodium dodecylsulphate (SDS)/DTT containing buffer, we used 10–13% linear gradient SDS-polyacrylamide gel electrophoresis (PAGE) in the second dimension. In parallel, we analysed 1 μg SP-A mixed with 10 μg total cell extract (from a breast cancer cell line, MDA-MB-231) containing proteins with known identity, isoelectric point (pI) and molecular weight. After fixation and silver staining [16], gels were scanned (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) and processed using the PDQUESTTM software (PDI, Huntington Station, NY, USA).

Preparation of modified natural surfactant. Modified porcine surfactant (equivalent to Curosurf) was isolated from minced pig lungs by a combination of washing, extraction with organic solvents, and liquid-gel chromatography [17]. The final preparation contained approximately 99% polar lipids, mainly phospholipids, and about 1% hydrophobic proteins (SP-B and SP-C in approximate molar proportions 1:2). PC constituted about 74% of the phospholipids, and about 46% of the PC fraction was DPPC. For animal experiments, the surfactant was suspended in 0.9% NaCl at a concentration of either 80 or $40 \text{ mg}\cdot\text{mL}^{-1}$.

Reconstitution of surfactant with SP-A. Purified SP-A was dissolved in 0.9% NaCl at a concentration of $5 \text{ mg}\cdot\text{mL}^{-1}$, and mixed with Curosurf and CaCl_2 , so that the final reconstituted surfactant contained $40 \text{ mg}\cdot\text{mL}^{-1}$ of phospholipid, $2 \text{ mg}\cdot\text{mL}^{-1}$ of SP-A (5% by weight) and 2 mM CaCl_2 . This mixture (SP-A-Curosurf) was gently mixed in a rocking plate apparatus for 30 min at room temperature and then frozen at -20°C .

Surface tension measurements

For surface tension measurements, the surfactant preparations were diluted to a phospholipid concentration of either 5 or $1 \text{ mg}\cdot\text{mL}^{-1}$. Surface properties were determined in a pulsating bubble system (Surfactometer International, Toronto, Canada). Approximately 20 μL of the test fluid was filled in a plastic sample chamber and a bubble was created. The bubble was kept at static conditions for 2 min and then pulsed for 10 min at 37°C at a rate of $40 \text{ pulses}\cdot\text{min}^{-1}$, with 50% cyclic compression of the bubble surface [18]. Pressure across the bubble wall was recorded continuously for 10 min. Values for surface tension at maximum and minimum bubble diameter (1.1 and 0.8 mm, respectively) were calculated according to the LaPlace equation: $P=2\gamma/r$, where P is the measured pressure gradient across the bubble wall, r the radius, and γ the surface tension. Equilibrium surface tension was determined 2–3 min after the

period of pulsation, while the bubble was kept at maximum size and no further change in the pressure gradient could be recorded. Mean values were obtained from 8–11 observations for each sample.

In vitro exposure to surfactant inhibitors. For these studies, Curosurf, SP-A-Curosurf and NPS were suspended with 0.9% NaCl at a concentration of 5 mg·mL⁻¹. Human meconium, collected from healthy newborns and processed as reported elsewhere [19], was added to a final concentration of 5 mg dry weight·mL⁻¹. Human fibrinogen and human albumin (both from Sigma Chemical Co., St Louis, MO, USA), were added to each of the three surfactant preparations at final concentrations of 5 and 25 mg·mL⁻¹, respectively. Rabbit serum (protein concentration, 70 mg·mL⁻¹), prepared at our laboratory, was added at a final protein concentration of 25 mg·mL⁻¹. In all these surfactant and surfactant-protein samples, CaCl₂ was added at a concentration of 2 mM, and NaCl was kept at 0.9%.

Effects of EDTA. In order to determine whether the effects of SP-A were calcium ion dependent, ethylenediamine tetra-acetic acid (EDTA) was added to the samples at a final concentration of 3 mM. Surface tension measurements were then performed on Curosurf, SP-A-Curosurf, and NPS mixed with rabbit serum or fibrinogen as described above. Excess CaCl₂ (10 mM) was added to some samples of SP-A-Curosurf, mixed with fibrinogen or rabbit serum, to counterbalance the effects of EDTA.

Animal experiments

The animal experiments were designed to test the specific hypothesis that addition of SP-A to Curosurf administered at a dose of 100 mg·kg⁻¹ would increase the dose-effectiveness of the preparation in newborn animals with surfactant deficiency or dysfunction, and make the therapeutic response similar to that obtained with natural surfactant at the same dose. The protocols for these experiments were approved by the local Ethics Committee for experimental animal use.

New Zealand White rabbit foetuses were delivered by hysterotomy at a gestational age of either 27 or 29 days (term = 31 days). The lungs of the 27 day animals were immature and surfactant-deficient, those of the 29 day animals were nearly mature. At delivery, all the foetuses were anaesthetized with intraperitoneal sodium pentobarbital (0.1 mL; 6 mg·mL⁻¹), tracheotomized, paralysed with intraperitoneal pancuronium bromide (0.1–0.15 mL; 0.2 mg·mL⁻¹), and kept in a ventilator-multiplethysmograph system at 37°C. They were ventilated in parallel with a modified Servo-Ventilator (900B, Siemens-Elcoma, Solna, Sweden) delivering 100% oxygen at a frequency of 40 breaths·min⁻¹ and 50% inspiration time. No positive end-expiratory pressure (PEEP) was applied. Tidal volume (VT) was set at approximately 10 mL·kg body weight⁻¹ by individual adjustment of peak inspiratory pressure (PIP) [20]. Dynamic lung-thorax compliance was derived from recordings of VT and PIP and expressed as mL·cmH₂O⁻¹·kg⁻¹.

Protocol I. The immature newborn rabbits were randomized to receive at birth, *via* the tracheal cannula, 2.5

mL·kg⁻¹ of SP-A-Curosurf (40 mg·mL⁻¹), Curosurf (40 or 80 mg·mL⁻¹), or NPS (40 mg·mL⁻¹). In control animals, no material was instilled into the airways. All animals were ventilated for 60 min. Compliance was measured at 15, 30, 45 and 60 min.

Protocol II. The nearly mature newborn rabbits were first ventilated for 15 min, and animals with lung-thorax compliance ≥ 0.45 mL·cmH₂O⁻¹·kg⁻¹ were then randomly assigned to six groups. The control group (Group 1) received 4 mL·kg⁻¹ of 0.9% saline intratracheally, and the rest of the animals (Groups 2–6) received, by the same route, 4 mL·kg⁻¹ of human fibrinogen (20 mg protein·mL⁻¹). Forty five minutes after the fibrinogen instillation, these animals were treated with intratracheal instillation of 2.5 mL·kg⁻¹ of either 0.9% saline (Group 2), SP-A-Curosurf (Group 3), Curosurf, 40 mg·mL⁻¹ (Group 4), Curosurf 80 mg·mL⁻¹ (Group 5) or NPS (Group 6), and ventilated for another 120 min. Compliance was measured at 15, 30, 45, 60, 90, 120, 150 and 180 min.

Electrocardiograph (ECG) readings were recorded in both immature and nearly mature animals by means of subcutaneous electrodes. The ECG was checked at the same intervals as indicated above, and animals with arrhythmia or absence of QRS complexes were excluded from the final data analysis. At the end of the experiment, animals were sacrificed by intracranial injection of lidocaine (0.5 mL; 20 mg·mL⁻¹). The abdomen was opened and the diaphragm inspected for evidence of pneumothorax. Thereafter, the chest was opened, and blood was taken from the right ventricle of the heart for measurement of PCO₂ and pH.

The lungs of all animals were inflated with a pressure of 30 cmH₂O for 1 min. Transpulmonary pressure was then lowered to 10 cmH₂O, which was maintained for 30 min while the lungs were perfused *via* the pulmonary arteries with 3.8% formaldehyde at a pressure of 65 cmH₂O. The whole lungs were embedded in paraffin, and transverse sections from all lobes were stained with haematoxylin and eosin. The sections were examined by light microscopy, and volume density (Vv) of alveolar spaces was determined by conventional point counting, using total parenchyma as reference volume [21].

Statistical analysis

Values are presented as means and standard deviations (SD). Differences between groups were examined by analysis of variance (ANOVA) followed by Student-Newman-Keuls' *post-hoc* test. Differences within groups were examined by Wilcoxon signed-ranks test. The Pearson method was used for correlation of physiological and histological data. A p-value of less than or equal to 0.05 was regarded as statistically significant.

Results

Isolation of SP-A

Isolated porcine SP-A appeared as a single peak on HPLC (fig. 1), corresponding to a molecular weight of

about 650 kDa (co-elution with thyroglobulin, monomer 669 kDa). The reduced protein could be separated by 2-DE into more than six major isoforms with pI ranging 4.0–4.5 and molecular weights of 28–40 kDa (fig. 2).

Surface tension measurements

Surface properties of Curosurf, SP-A-Curosurf, and NPS at two different phospholipid concentrations are shown in table 1. At a concentration of 5 mg·mL⁻¹, the

Fig. 1. – Chromatography of surfactant protein A (SP-A) on a Novarose™ SE-1000/17, 8 × 300 mm column. The void volume (V_0) and the total volume (V_{tot}) were determined by the dimer of thyroglobulin and acetone, respectively (not shown).

Fig. 2. – Purified porcine surfactant protein A (SP-A) separated by 2-dimensional isoelectric-focusing electrophoresis as described in Materials and methods. Identified isoforms of SP-A have an isoelectric point range of approximately 4.0–4.5.

Table 1. – Surface properties of different surfactant preparations tested at 37°C in a pulsating bubble system

Surfactant	Conc. mg·mL ⁻¹	Surface tension mN·m ⁻¹		
		Minimum	Maximum	Equilibrium
Curosurf	5	3.2±1.5	35.1±1.8	25.6±0.6
	1	16.3±3.9**	48.4±8.4**	33.4±7.6**
SP-A-Curosurf	5	5.5±3.9	33.9±1.1	25.9±0.9
	1	8.4±3.6***	36.9±3.3***	25.9±0.6##
NPS	5	4.0±2.9	33.8±0.9	24.3±0.4
	1	7.5±5.5##	34.8±2.6##	25.4±0.8***

Values were obtained after 10 min pulsation, and are presented as mean±SD, n=8–11. Conc.: concentration; SP-A: surfactant protein A; NPS: natural porcine surfactant. *, **, p<0.05, p<0.01, vs the same surfactant at higher concentration; ##, p<0.01, vs Curosurf, 1 mg·mL⁻¹.

Fig. 3. – Dynamic surface tension of various surfactant preparations exposed to different inhibitions. All preparations contained 5 mg·mL⁻¹ surfactant, in addition to: a) 5 mg·mL⁻¹ meconium; b) 5 mg·mL⁻¹ fibrinogen; c) 25 mg·mL⁻¹ albumin; d) 25 mg·mL⁻¹ serum protein. CaCl₂ (2 mM) was present in all samples. Values for maximum and minimum surface tension were recorded after 10 min of cyclic area compression in a pulsating bubble system. □: Curosurf; ▨: surfactant protein A (SP-A)-Curosurf; ▤: natural porcine surfactant (NPS). *, **, p<0.05, p<0.01 vs SP-A-Curosurf and NPS.

three preparations were similar. However, when the surfactants were diluted to $1 \text{ mg}\cdot\text{mL}^{-1}$, values for minimum, maximum, and equilibrium surface tension were significantly higher for Curosurf than for each of the other two preparations.

Inhibitory effects of meconium, fibrinogen, albumin,

Fig. 4. — Maximum and minimum surface tension of Curosurf, natural porcine surfactant (NPS) and fibrinogen ($5 \text{ mg}\cdot\text{mL}^{-1}$), and NPS and serum protein ($25 \text{ mg}\cdot\text{mL}^{-1}$) in the presence of 2 mM CaCl_2 and 3 mM ethylenediamine tetra-acetic acid (EDTA). The concentration of surfactant was the same in all samples ($5 \text{ mg}\cdot\text{mL}^{-1}$). Values were recorded after 10 min of surface area oscillation in a pulsating bubble system. □: Curosurf; ▨: NPS and fibrinogen; ▩: NPS and serum. **: $p < 0.01$, vs Curosurf, and NPS and fibrinogen; ++: $p < 0.01$, vs Curosurf.

Fig. 5. — Maximum and minimum dynamic surface tension of surfactant protein A (SP-A)-Curosurf ($5 \text{ mg}\cdot\text{mL}^{-1}$) exposed to serum proteins ($25 \text{ mg}\cdot\text{mL}^{-1}$) or fibrinogen ($5 \text{ mg}\cdot\text{mL}^{-1}$) in the presence of 3 mM ethylenediamine tetra-acetic acid (EDTA) and different concentrations of CaCl_2 . □: serum protein and 2 mM CaCl_2 ; ▨: fibrinogen and 2 mM CaCl_2 ; ▩: serum protein and 10 mM CaCl_2 ; ▪: fibrinogen and 10 mM CaCl_2 . **: $p < 0.01$, vs 10 mM CaCl_2 .

and serum proteins, studied at a standard surfactant concentration ($5 \text{ mg}\cdot\text{mL}^{-1}$) are shown in figure 3. Curosurf was inhibited by all these substances, as shown by significantly elevated values for both maximum and minimum surface tension. In contrast, values for maximum and minimum surface tension of SP-A-Curosurf and NPS were not affected by any of these inhibitors.

Addition of EDTA had no adverse effects on Curosurf but provoked inactivation of NPS exposed to serum, as reflected by significantly increased levels for both maximum and minimum surface tension (fig. 4). EDTA increased maximum, but not minimum, surface tension of NPS exposed to fibrinogen (fig. 4). SP-A-Curosurf was inactivated by serum and fibrinogen when EDTA was added to the system, but this inactivation could be reversed by increasing the calcium concentration to 10 mM (fig. 5).

Animal experiments

Immature animals (Protocol I). Data from these experiments are summarized in table 2. Fifty eight of the 76 animals enrolled in the study survived the period of ventilation without complications. Eighteen animals were excluded from further analysis because of pneumothorax ($n=10$) or ECG abnormalities ($n=8$). There was no difference in the incidence of these complications between the groups. Body weight and final cardiac frequency were similar in all groups of animals surviving without complications. Tidal volumes at the end of the experiments were very close to $10 \text{ mL}\cdot\text{kg}^{-1}$, as defined in the protocol (data not shown). In comparison with untreated controls, compliance was improved in all surfactant-treated groups, especially in animals receiving NPS. Values for P_{CO_2} in heart blood were significantly reduced, and alveolar Vv in histological sections significantly increased in animals receiving NPS or high doses of Curosurf ($80 \text{ mg}\cdot\text{mL}^{-1}$; $200 \text{ mg}\cdot\text{kg}^{-1}$). The latter group also had the highest mean value for pH in heart blood. There were no differences in lung-thorax compliance, blood gases or alveolar Vv between animals receiving SP-A-Curosurf and those treated with Curosurf alone. In the material as a whole, final compliance was significantly correlated to alveolar Vv ($r=0.69$; $p < 0.001$).

Near-term animals (Protocol II). These findings are summarized in tables 3 and 4. Nine animals were excluded from the study because compliance at 15 min was $\leq 0.45 \text{ mL}\cdot\text{cmH}_2\text{O}^{-1}\cdot\text{kg}^{-1}$. Seventy three of the 80 animals enrolled in the subsequent experiments survived the scheduled

Table 2. — Incidence of complications, body weight, lung-thorax compliance, final cardiac frequency (f_c) CO_2 tension (P_{CO_2}) and pH in heart blood after 60 min of ventilation, and alveolar volume density (Vv) in immature newborn rabbits (Protocol I)

Group	n	Pneumo/ECG abnormalities n/n	Body weight g	Lung-thorax compliance $\text{mL}\cdot\text{cmH}_2\text{O}^{-1}\cdot\text{kg}^{-1}$	Final f_c $\text{beats}\cdot\text{min}^{-1}$	P_{CO_2} kPa	pH	Alveolar Vv
1. Control	12	1/0	31.1 ± 5.7	0.33 ± 0.06	207 ± 34	9.4 ± 1.9	6.89 ± 0.17	0.19 ± 0.08
2. SP-A-Curosurf	12	3/2	29.6 ± 6.4	$0.48 \pm 0.10^{**}$	224 ± 36	7.9 ± 1.8	7.01 ± 0.13	$0.27 \pm 0.06^*$
3. Curosurf $100 \text{ mg}\cdot\text{kg}^{-1}$	13	1/0	28.9 ± 4.1	$0.44 \pm 0.11^*$	216 ± 41	8.0 ± 1.8	7.00 ± 0.16	$0.26 \pm 0.07^*$
4. Curosurf $200 \text{ mg}\cdot\text{kg}^{-1}$	11	2/4	31.5 ± 5.8	$0.51 \pm 0.12^{**}$	228 ± 44	$6.9 \pm 1.8^{**}$	$7.12 \pm 0.10^{**}$	$0.33 \pm 0.08^{**}$
5. NPS	10	3/2	30.0 ± 5.2	$0.57 \pm 0.15^{***}$	193 ± 21	$7.3 \pm 1.2^*$	7.04 ± 0.14	$0.43 \pm 0.9^{***}$

Values are presented as mean $\pm$ SD. *, **: $p < 0.05$, $p < 0.01$, vs Group 1; #: $p < 0.01$, vs Groups 2, 3 and 4. Pneumo: pneumothorax; ECG: electrocardiogram. For further definitions, see table 1.

Table 3. – Incidence of complications, body weight, fc , PCO_2 and pH in heart blood after 3 h of ventilation, and alveolar V_V in ventilated nearly mature newborn rabbits (Protocol II)

Group	n	Pneumo/ECG abnormalities n/n	Body weight g	Final fc beats·min ⁻¹	PCO_2 kPa	pH	Alveolar V_V
1. Saline+saline	12	0/0	40.2±4.7	233±29	6.8±1.5	7.25±0.12	0.39±0.13
2. F+saline	14	0/1	40.4±4.8	237±39	7.5±2.3	7.15±0.17	0.28±0.13
3. F+SP-A-Curosurf	14	0/1	43.6±6.1	237±30	7.1±1.2	7.22±0.07	0.38±0.08
4. F+Curosurf 100 mg·kg ⁻¹	12	0/2	39.6±6.7	223±25	7.3±1.5	7.20±0.10	0.40±0.12
5. F+Curosurf 200 mg·kg ⁻¹	11	0/1	40.7±5.6	230±24	6.1±1.3	7.24±0.17	0.36±0.13
6. F+NPS	10	0/2	39.5±6.5	217±45	7.8±2.3	7.15±0.16	0.39±0.14

Fibrinogen (F)/saline was given after 15 min, and surfactant/saline after 60 min. Values are presented as mean±SD. There were no statistically significant differences between the groups. For further definitions, see tables 1 and 2.

period of ventilation without complications. Seven animals were excluded from further analysis because of ECG abnormalities; there was no difference in the incidence of this complication between the groups. Body weight and final cardiac frequency in animals surviving without complications showed no difference between the groups. The same was true for PCO_2 and pH in heart blood obtained at the end of the experiment. In comparison with animals receiving fibrinogen and saline, compliance at 120 min was significantly improved only in the group treated with SP-A-Curosurf (table 4). At 180 min, compliance was improved in all surfactant-treated animals, with no difference between the groups. Statistical analysis of differences within groups revealed that administration of fibrinogen at 15 min caused a significant fall in compliance in all animals, with further deterioration at subsequent time intervals in those not receiving surfactant. After treatment with surfactant at 60 min, compliance improved significantly in animals receiving SP-A-Curosurf, both at 120 and 180 min, but was not restored to normal. In animals treated with high dose of Curosurf (80 mg·mL⁻¹; 200 mg·kg⁻¹), the improvement in compliance was significant only in the final recordings at 180 min, but again normal values were not established. Animals receiving the lower dose of Curosurf (40 mg·mL⁻¹; 100 mg·kg⁻¹) did not improve after surfactant therapy. In animals receiving saline only (Group 1), there was an initial significant fall in compliance followed by a gradual improvement, and the values obtained at 180 min were significantly higher than those recorded at 60 min.

Histological examination of lungs from Protocol II animals receiving saline only (Group 1) showed a uniform alveolar expansion pattern and well-preserved epithelium in conducting airways. Animals receiving fibrinogen without subsequent surfactant treatment had widespread

atelectasis, associated with varying degrees of intra-alveolar oedema, hyaline membranes, recruitment of neutrophils to the airspaces, and desquamation of airway epithelium. Similar, but less prominent abnormalities were observed in animals treated with different surfactant preparations, without obvious differences between the groups. Alveolar V_V was significantly lower in animals receiving fibrinogen and saline than in each of the other groups, but did not differ between animals receiving different preparations of surfactant (table 3). In the material as a whole, there was a significant correlation between compliance at 180 min and alveolar V_V ($r=0.55$; $p<0.001$).

Discussion

Physiologically active surfactants for clinical use can be prepared from animal lungs by extraction of natural surfactant with organic solvents. Such "lipid extract surfactants" contain small amounts of hydrophobic proteins (SP-B, SP-C) that seem to be essential for their biophysical effects [22]. SP-A (as well as the other hydrophilic surfactant protein, SP-D) is absent in these surfactants, which adsorb rapidly to an air-liquid interface without forming tubular myelin as an intermediate phase [23]. Our present data show that the physical properties of Curosurf at low concentration are improved by adding SP-A. At a Curosurf concentration of 1 mg·mL⁻¹, the presence of 5% SP-A reduced maximum, minimum and equilibrium surface tension in a pulsating bubble system, indicating more effective adsorption and increased film stability during surface compression. At a Curosurf concentration of 5 mg·mL⁻¹, addition of SP-A made no difference in the absence of inhibitors, and values for dynamic surface tension of

Table 4. – Lung-thorax compliance at various time intervals in ventilated nearly mature newborn rabbits (Protocol II)

Group	n	Compliance mL·cmH ₂ O ⁻¹ ·kg ⁻¹				Within-group differences p-value					
		15 min (C15)	60 min (C60)	120 min (C120)	180 min (C180)	C60– C15	C120– C15	C180– C15	C120– C60	C180– C60	C180– C120
1	12	0.67±0.23	0.54±0.11	0.57±0.14*	0.61±0.16**	<0.05				<0.01	<0.05
2	14	0.70±0.18	0.49±0.08	0.43±0.10	0.39±0.11	<0.01	<0.01	<0.01		<0.01	<0.05
3	14	0.75±0.29	0.50±0.11	0.57±0.10*	0.57±0.10**	<0.01	<0.05	<0.05	<0.01	<0.01	
4	12	0.78±0.20	0.48±0.14	0.52±0.11	0.50±0.12*	<0.01	<0.01	<0.01			
5	11	0.66±0.22	0.48±0.07	0.53±0.09	0.56±0.08*	<0.05	<0.05			<0.05	
6	10	0.60±0.18	0.46±0.12	0.52±0.15	0.53±0.17*	<0.05	<0.05				

*, **: $p<0.05$, $p<0.01$, vs Group 2. For definitions of groups, see table 3.

Curosurf without SP-A were the same as for natural surfactant isolated by sucrose-gradient centrifugation.

Differences between surfactants with or without SP-A became apparent at the higher concentration (5 mg·mL⁻¹) when various inhibitors were added to the system. Our data clearly demonstrate that the presence of SP-A increases the resistance to inactivation by meconium, fibrinogen, albumin, and total serum proteins. These observations, which are in agreement with data on bovine lipid extract surfactant enriched with SP-A [6], have potential clinical implications. Endogenous as well as exogenous surfactant may become exposed to these inhibitors in, for example, meconium aspiration syndrome and in inflammatory conditions with increased alveolar microvascular permeability such as ARDS.

In our experiments on immature newborn rabbits, we found no significant physiological effect of SP-A added to Curosurf administered at a dose of 100 mg·kg⁻¹. A similar negative result was obtained by YAMADA *et al.* [9] in their study evaluating the effect of 5% SP-A added to Surfactant given to ventilated immature rabbits at a dose of 50 mg·kg⁻¹. On the other hand, adding 2–10% SP-A to an organic solvent extract of natural surfactant administered at the same dose clearly improved the therapeutic effect, and values for lung-thorax compliance were similar to those in animals treated with natural surfactant [9]. Thus, the physiological effects of added SP-A may vary depending on the composition of the starting material. Surfactant and Curosurf are both different from an ordinary lipid extract surfactant, Surfactant being fortified with synthetic lipids, and Curosurf being purified by liquid-gel chromatography after the extraction procedure.

In immature newborn animals, the magnitude of compliance improvement after surfactant therapy may also depend on the style of ventilation. Suboptimal surfactant preparations require ventilation with PEEP to exert a measurable therapeutic effect, whereas immature animals receiving complete natural surfactant can be ventilated effectively without PEEP [24]. In the present experiments, we chose to ventilate all animals without PEEP to increase our chances of detecting differences in quality between various surfactant preparations. The compliance levels of both surfactant-treated and control animals shown in table 2 are generally lower than those reported in our original paper validating the animal model [20], suggesting that, although gestational age and birth weight were nearly the same, the animals recruited to the present experiments were studied at a slightly earlier stage of pulmonary development.

The inhibitory effect of fibrinogen on surfactant function has been demonstrated under *in vitro* conditions by several investigators [6, 25–27]. The animal model used in the present study represents a new approach to experimental evaluation of exogenous surfactants with particular reference to their resistance to inactivation by serum proteins. In previous experiments, we used a similar method to study the effects of a monoclonal antibody to SP-A. We found that administration of the antibody *via* the airways did not in itself interfere with lung function [28], in accordance with earlier data published by KOBAYASHI *et al.* [29]. However, when the animals were challenged with intratracheal instillation of fibrinogen, the fall in compliance and degree of lung

injury were more prominent in animals receiving the antibody to SP-A. In the present study, the animals were first exposed to fibrinogen, which caused a mean reduction in compliance of 30% (pooled data from Groups 2–6). We then evaluated the response to treatment with different surfactant preparations. Our data indicate that the presence of SP-A potentiated the treatment effect when the surfactant was given at a low dose (100 mg·kg⁻¹). The physiological effects of the higher dose of Curosurf (200 mg·kg⁻¹) were similar to those obtained with NPS (100 mg·kg⁻¹), suggesting that accumulation of inhibitors in the airspaces can be counterbalanced not only by the presence of SP-A but also by increasing the pool of surfactant lipids and hydrophobic proteins in the airspaces.

In summary, addition of surfactant protein A improves the surface properties of Curosurf at low concentration and increases resistance to inactivation by plasma proteins. The presence of surfactant protein A also improves the dose-effectiveness of the exogenous surfactant in near-term newborn rabbits with respiratory failure induced by airway instillation of fibrinogen. We speculate that the requirement for large doses of lipid extract surfactants in clinical practice may reflect a comparatively high sensitivity to inactivation, and that the use of exogenous surfactant enriched with surfactant protein A might be considered in certain clinical conditions characterized by the flooding of alveoli with plasma proteins.

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