ORIGINAL ARTICLES

Surfactant improves lung function and mitigates bacterial growth in immature ventilated rabbits with experimentally induced neonatal group B streptococcal pneumonia

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Abstract

Aims—To study the influence of surfactant on lung function and bacterial proliferation in immature newborn rabbits with experimental group B streptococcal (GBS) pneumonia.

Methods—Preterm rabbit fetuses (gestational age 28 days) underwent tracheotomy and were mechanically ventilated in a warmed body plethysmograph that permitted measurement of lung-thorax compliance. Fifteen minutes after the onset of ventilation the animals received either GBS or saline intratracheally; at 30 minutes, a bolus of saline or 200 mg/kg of a porcine surfactant (Curosurf) was administered via the airway. Bacterial proliferation was evaluated in lung homogenate at the end of the experiments and the results expressed as mean log₁₀ cfu/g lung (SD). Animals receiving only saline (n=20) or saline and surfactant (n=20) served as controls.

Results—The average survival time was about three hours in all groups. Infected animals receiving surfactant (n = 22) had significantly less bacterial growth (9.09 (0.45) vs 9.76 (0.91)) and improved lung function (compliance: 0.61 (0.14) vs 0.34 (0.19) ml/kg . cm H₂O) than infected rabbits receiving saline at 30 minutes (n = 22). **Conclusion**—Surfactant improves lung function and mitigates bacterial growth in preterm rabbits infected with group B streptococci.

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Keywords: surfactant; rabbits; group B streptococci; lung function; bacterial growth.

Group B streptococci (GBS) cause considerable mortality and morbidity in the neonatal period. Vaginal colonisation with this organism is common during pregnancy.¹ One to four in every thousand newborn infants has early onset GBS septicaemia. The incidence is higher in premature newborn babies and although only a

few term newborn infants die from the disease nowadays, there is a disturbingly high mortality in premature babies with GBS infections.^{1,2}

Most premature infants with systemic or pulmonary GBS infections have respiratory symptoms.^{2,3} Thus many of these infants are likely to be given surfactant treatment before the final diagnosis is established. There has been considerable concern that surfactant treatment might actually harm these infants, as previous animal studies have shown an increased bacterial growth following surfactant treatment in immature rabbit fetuses infected with aerosolised GBS.⁴ In addition, decreased phagocytosis and a diminished release of cytokines, neutrophil elastase, and oxygen metabolites have been described following in vitro incubation of phagocytes with surfactant.^{5,6}

Recently, we identified a GBS strain that is pathogenic to rabbits and developed an animal model to study the effect of surfactant in ventilated near-term newborn rabbits following intratracheal infection with GBS.⁷ However, in a clinical situation term newborn infants would rarely receive surfactant treatment for respiratory failure due to pneumonia, the main target group for surfactant replacement being premature infants with a combination of surfactant deficiency and pulmonary infection.

The present study aimed to evaluate the effect of surfactant treatment on lung function, inflammatory changes, and bacterial growth in immature ventilated newborn rabbits with experimentally induced neonatal GBS pneumonia.

Methods

BACTERIA

An abundantly encapsulated low density (LD) phase variant of bacteria was processed from the reference strain GBS 090 Ia Colindale by repeated gradient centrifugation (kind gift of Stellan Håkansson, University of Umeå, Sweden). Previous experience had indicated that the polysaccharide capsule is an important pathogenic factor in rabbits.⁸ GBS type Ia is a

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subtype commonly isolated from neonates with early onset group B streptococcal septicaemia; type III is commonly found in neonates with late onset disease, especially meningitis. The strain was stored in aliquots at -70°C , precultured, washed, centrifuged and suspended in physiological saline at a concentration of 10^9 live bacteria per ml. The number of colony forming units (cfu) in the stock suspension was determined for each individual experiment by serial dilution and bacterial counting on blood agar plates following a 24 hour incubation period at 37°C with 5% CO_2 . Details of the procedure have been described elsewhere.⁷

SURFACTANT

Curosurf is a modified natural surfactant isolated from minced pig lungs. Neutral lipids, cholesterol, and lipid esters are removed by a combination of chloroform-methanol extraction and liquid-gel chromatography. It is sterilised by high pressure filtration through a micropore filter system and finally suspended at a concentration of 80 mg/ml of phospholipids.⁹ Apart from 99% polar lipids (~35% dipalmitoylphosphatidylcholine), it contains about 1% of proteins. The water-soluble surfactant proteins SP-A and SP-D are removed by the extraction procedure; only the hydrophobic proteins SP-B and SP-C, which are of major importance for adequate surfactant function, are contained in the preparation. Curosurf is effective *in vitro*, in animal experiments, and in controlled randomised clinical trials for the treatment of respiratory distress syndrome (RDS).⁹

ANIMAL EXPERIMENTS

Preterm rabbit fetuses (New Zealand White) were delivered at a gestational age of 28 days by caesarean section. Term gestation in rabbits is 31 days. The animals were anaesthetised and underwent tracheotomy at birth, after which they were transferred to a warmed plethysmograph system,¹⁰ as described before.⁷ They were mechanically ventilated in parallel in sealed Plexiglass chambers with a common ventilator system (Servo 900 B, Siemens-Elema, Solna, Sweden) delivering 100% oxygen. The working (maximum) pressure was set at 50 cm H_2O . The frequency was 40 per minute, the inspiration:expiration time ratio 1:1. No positive end-expiratory pressure (PEEP) was applied, as PEEP might mask differences in compliance due to variations in surfactant function.¹¹ The peak inspiratory pressure was recorded with a pressure transducer (EMT 34) and individually adjusted for each animal to obtain a tidal volume of 8-10 ml/kg bodyweight. Tidal volume was recorded with a specially designed "Fleisch-tube," a differential pressure transducer (EMT 31), an integrator (EMT 32), an amplifier (EMT 41) and a recording system (Mingograf 81; all equipment, Siemens-Elema). The system was calibrated for each individual experiment and a linear calibration curve was obtained for tidal volumes between 0.1 and 0.8 ml. Lung-thorax compliance (ml/kg . cm H_2O) was calculated from the quotient of tidal volume (expressed in ml/kg) and

peak inspiratory pressure (expressed in cm H_2O). All recordings were obtained at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270 and 300 minutes. An electrocardiogram (ECG) was recorded at the same intervals and animals were counted as survivors if the heart rate was >100 per minute without evidence of arrhythmia or atrioventricular block.

At birth the animals were randomly allocated to the different treatment groups. At 15 minutes they received an intratracheal injection of either the GBS suspension or sterile NaCl 0.9% (5 ml/kg). At 30 minutes, either surfactant (Curosurf, 2.5 ml/kg) or again saline (2.5 ml/kg) was administered via the tracheal cannula. The experimental groups that originated from this procedure were as follows.

Animals infected with GBS and treated with either saline (GBS/NaCl) or Curosurf (GBS/Curosurf) and animals treated initially with saline receiving saline again (NaCl/NaCl) or the same volume of Curosurf (NaCl/Curosurf). The maximum period of ventilation was 300 minutes in all these animals. Animals in a fifth group were infected at 15 minutes with 5 ml/kg GBS and ventilated for 1 minute to move the instilled bacteria from the dead space to the periphery of the lungs. This group (GBS/1 min) served as a baseline for the evaluation of bacterial numbers and inflammatory changes. The GBS/NaCl and the GBS/Curosurf groups were used for the direct evaluation of the effect of surfactant on bacterial proliferation. The saline group (NaCl/NaCl) served as a control group for comparison with the NaCl/Curosurf group, especially as regards the influence of surfactant on lung function.

At the end of the experiments the animals were killed by intracranial injection of xylocaine 0.5%; the chest was opened with sterile instruments after the diaphragm had been examined for evidence of pneumothorax. Blood from the right cardiac ventricle was aspirated for a blood culture (Bactec Plus blood culture system, Becton Dickinson, Sparks, Maryland, USA). A sample anticoagulated with heparin was taken for blood gas analysis. The left lung was excised and cut into two halves in the sagittal plane. The peripheral part of the lung was weighed, placed immediately into the sterilised tube of a tissue homogeniser (Kontes Scientific Glassware instruments, Vineland, New Jersey, USA), and stored on ice until further processing. The medial part of the left lung was weighed and fixed for histological examination (see below). The right lung was used for biochemical studies not included in this report.

BACTERIAL COUNTING

The lung specimens were preprocessed with a pellet pestle dispenser (Kontes Scientific Glassware) and the weight of the sample was adjusted to 1 g with sterile NaCl 0.9%. The lung was homogenised with a high speed (15000 rpm for 60 seconds) nylon microchamber tissue homogeniser (Sorval Omnimixer, Dupont Instruments, Newton, Connecticut, USA). Lung homogenate (0.5 ml) was sus-

Table 1 Characterisation of animals in the experimental groups; values are mean (SD)

Parameter	Experimental group				
	GBS/1 minute (n=14)	GBS/NaCl (n=22)	GBS/Curosurf (n=22)	NaCl/NaCl (n=20)	NaCl/Curosurf (n=20)
Treatment 15 minutes	GBS 5 ml/kg	GBS 5 ml/kg	GBS 5 ml/kg	NaCl 5 ml/kg	NaCl 5 ml/kg
Treatment 30 minutes	Sacrificed at 16 minutes	NaCl 2.5 ml/kg	Curosurf 2.5 ml/kg	NaCl 2.5 ml/kg	Curosurf 2.5 ml/kg
Body weight (g)	35 (7)	37 (5)	35 (6)	36 (7)	36 (8)
Left lung weight (g)	0.40 (0.11)	0.32 (0.08)	0.35 (0.09)	0.31 (0.07)	0.34 (0.09)
Final survival time (minutes)	16 (0)	184 (93)	174 (105)	189 (90)	184 (108)
Heart rate (beats/minute)	195 (88)	74 (68)	129 (98)	111 (97)	144 (108)
pCO ₂ (kPa)	11.2 (3.6)	8.3 (4.1)	7.5 (2.9)	7.5 (3.6)	7.8 (4.3)

pended and mixed thoroughly in tubes with 4.5 ml of sterile saline. A serial dilution was performed down to 10^{-9} , and 100 μ l of the diluted bacterial suspensions were spread on blood agar plates. Colony counting was performed after 24 hour incubation, as described above. As bacterial proliferation follows a logarithmic growth curve the results were expressed as mean $\log_{10}$ cfu/g lung (wet weight).

HISTOLOGICAL EXAMINATION OF THE LUNGS

At the end of the experiment the medial part of the left lung was excised, fixed in formaldehyde 4 %, and subsequently embedded in paraffin wax. Transverse sections, stained with haematoxylin and eosin and Gram stain, were examined by light microscopy with special reference to the presence of intra-alveolar oedema, hyaline membranes, epithelial necrosis, bacterial proliferation and recruitment of inflammatory cells to the air spaces. The slides were coded so that the investigator was unaware of the experimental conditions of the individual animals.

STATISTICS

Data are given as mean (SD). Values for lung weight and physiological data were subjected to analysis of variance (ANOVA) using the CRISP software programme (Crunch Software, San Francisco, California, USA). Inter-group differences were evaluated using Student's and Newman-Keuls's tests. Differences in the incidence of complications between the groups were analysed using the χ^2 test. The limit of significance was defined as $P = 0.05$.

The study design and the management of the animals complied with national legislation. The trial protocol was approved by the local committee for animal research.

Results

Ninety six fetuses were included in the data analysis. One animal per litter (n= 14) served as a control for bacterial growth at the beginning of the experiments (GBS/1min). Mean survival time was about 3 hours in all the other groups (table 1). Mean birthweight, left lung weight, final heart rate and pCO₂ were not significantly different between the groups (table 1).

A similar dose of bacteria was given to all three infected groups at 15 minutes (fig 1A). Over the study period there was prominent bacterial proliferation in both the GBS infected groups treated with or without surfactant (fig 1B). However, significantly fewer bacteria were detected in the lungs of infected rabbits treated with surfactant than in those receiving saline at the same time point (fig 1B). All animals in the GBS/NaCl and 21 of 22 (95%) rabbits in the GBS/Curosurf group had GBS positive blood cultures at the end of the experiments.

At 15 minutes, no significant differences in compliance were found between the groups. Mean compliance was about 0.6 ml/kg . cm H₂O, representing an intermediate level between values for very immature (gestational age 27 days) and near term (29.5 days) rabbits.

Figure 1 (A) Bacterial inoculum injected intratracheally in different study groups. (B) Bacterial proliferation at the end of the experiment. GBS/1 min animals represent the number of GBS at the beginning of the experiment (cfu = colony forming units).

Figure 2 Lung-thorax compliance in different experimental groups at the beginning of the experiment (15 minutes) and after three hours of mechanical ventilation (180 minutes).

The large SD indicates considerable variations in lung maturity among the animals (fig 2). Within three hours a substantial drop in compliance occurred in the GBS/NaCl and the NaCl/NaCl groups (fig 2). Infected animals not receiving surfactant had the lowest compliance values ($P < 0.05$ vs GBS/Curosulf and NaCl/Curosulf). The highest compliance values were found in the NaCl/Curosulf group, indicating that some of these animals had surfactant deficiency based on lung immaturity and actually benefited from surfactant treat-

Figure 3 Histological lung section from an animal in the GBS/Curosulf group, showing prominent bacterial proliferation. (Gram staining.)

Figure 4 Histological lung section from an animal in the GBS/Curosulf group, showing uneven expansion and influx of phagocytes, especially neutrophils, into the alveolar spaces. (Haematoxylin and eosin.)

ment (fig 2). In infected animals receiving surfactant the compliance was significantly ($P < 0.05$) improved compared with infected litter mate controls receiving saline (fig 2).

HISTOLOGICAL FINDINGS

In the GBS/1 min group there was a uniform alveolar expansion pattern corresponding to that of the fluid filled fetal lung. In one of these rabbits (7%) a few phagocytic cells were present in the alveolar spaces; no inflammatory changes were found in the other animals. Few bacteria were detected by Gram staining. In contrast, prominent bacterial growth was found in the GBS infected groups ventilated for longer time periods (fig 3). Most of these lungs were unevenly expanded with epithelial necrosis, intra-alveolar oedema, and hyaline membrane formation. These findings were most prominent in the GBS/NaCl group. The inflammatory response varied, ranging from virtually no influx of inflammatory cells to severe pneumonia (fig 4) in the GBS infected groups. A severe inflammatory response involving more than 30% of the lung parenchyma was found in four animals in the GBS/NaCl group (18%) as opposed to one in 22 rabbits (5%) in the GBS/Curosulf group (not significant). In six of 20 (30%) of the non-infected animals receiving saline at 15 minutes, a mild influx of inflammatory cells was noted. In none of these animals was more than 10% of the lung parenchyma involved in the inflammatory reaction.

Discussion

Our experimental model closely mimics the clinical situation in neonatal GBS infection. Significant bacterial proliferation associated with septicaemia could be induced in more than 90% of the infected animals. Although mean survival time (3 hours) was 2 hours shorter than in previous experiments on near-term rabbits,⁷ the number of bacteria detected per gram of lung homogenate was higher in the preterm rabbits than in the mature animals infected with the same dose. Sherman *et al* described similar results, indicating that bacterial proliferation occurred more rapidly in immature rabbit pups infected with aerosolised GBS than in mature ones.^{12 13} These findings probably parallel the clinical situation. Necropsy findings from premature infants dying of GBS pneumonia often show overwhelming bacterial proliferation without a distinct inflammatory response. Similar to our results in the animal model, term newborn infants more often show bacterial proliferation that is accompanied by invasion of neutrophils into the lungs.^{14 15}

In a clinical situation it is often difficult to differentiate between RDS based on surfactant deficiency due to lung immaturity alone and RDS triggered by GBS infection.³ Intra-alveolar accumulation of leaking plasma proteins, bacterial degradation products, and substances released from neutrophils (such as oxygen free radicals, proteases including elastase) can result in a secondary surfactant dysfunction comparable with the acute respiratory distress syndrome (ARDS) seen in older

patients.¹⁶ In the present study we found a mild influx of neutrophils in some of the ventilated animals that were not infected with GBS, suggesting a possible role for an inflammatory process in the pathogenesis of RDS in preterm infants with primary surfactant deficiency.

In our experiments the animals infected with GBS had the lowest compliance values after 3 hours of mechanical ventilation. The administration of surfactant could at least in part restore lung function. The large dose of surfactant (200 mg/kg body weight) used in our experiments might be crucial in this context. We have recently shown, in an animal model of secondary surfactant dysfunction caused by experimental meconium aspiration, that an exogenous surfactant dose of 200 mg/kg resulted in a significant and sustained improvement in oxygenation whereas a relapse was observed following a dose of 100 mg/kg.¹⁷ Complete natural surfactants (human amniotic fluid surfactant, or surfactants prepared from lung lavage by sucrose gradient centrifugation) are probably superior in terms of resistance to surfactant inhibition.¹⁸ This can be attributed to the fact that these preparations contain larger amounts of SP-B and that the hydrophilic protein SP-A is present. In vitro data also indicate that SP-A and SP-D are important stimulants of macrophage function.¹⁹ Currently available surfactant preparations might therefore be suboptimal for treatment of congenital pneumonia.

However, SP-A seems to have a limited role in host defence against the "smooth" encapsulated phase variant of GBS used in our experiments. To stimulate phagocytosis this strain needs opsonisation by specific IgG antibodies.²⁰ A "complete" natural porcine surfactant (containing SP-A and SP-D) prepared by lung lavage and sucrose gradient centrifugation did not enhance bacterial killing in GBS infected near-term rabbits when compared with Curosurf at the same dose of 100 mg/kg.²¹ Furthermore, inactivation of endogenous SP-A by a monoclonal anti-SP-A antibody²² in near-term GBS infected rabbits did not provoke changes in lung function or bacterial proliferation compared with an identical dose of non-specific IgG administered intratracheally immediately after birth.²³

Data from clinical pilot studies^{24 25} suggest that surfactant can be used successfully in neonates with congenital pneumonia but that the response to surfactant treatment is slower than in babies with uncomplicated RDS.¹⁶ Although initial data from animal experiments published by Sherman and coworkers indicated that it might be harmful to treat infected neonates with surfactant,⁴ the same group recently published an investigation showing that various commercially available modified natural surfactant preparations do not promote bacterial growth.¹² In this study, using aerosolised streptococci in spontaneously breathing rabbit pups, the synthetic surfactant preparation Exosurf inhibited bacterial growth significantly. This is similar to our present data which show that surfactant treatment actually mitigates bacterial growth, perhaps by preventing

atelectasis and accumulation of proteins in the alveoli.¹⁶ Keeping the airways open throughout the respiratory cycle is probably important for effective mucociliary clearance. Surfactant is also an important factor in fluid homeostasis of the neonatal lung. As the experimental animals were subjected to a considerable intratracheal fluid load postnatally, increased resorption of lung liquid might have contributed to the improved lung function observed in surfactant treated animals.

Data from other groups also indicate that surfactant might have an important role in the down-regulation of an inflammatory response in the lung. In recent experiments we observed a decreased release of reactive oxygen metabolites following incubation of neutrophils with Curosurf.²⁰ In other experiments the tissue concentrations of neutrophilic elastase were significantly reduced in GBS infected near-term rabbits treated with surfactant compared with infected controls receiving saline at the same time point.²⁶ It has already been shown that the presence of free elastase activity in bronchial secretions of premature infants with RDS correlates with acute lung damage (pulmonary interstitial emphysema).²⁷ Furthermore, elastase can cause proteolytic damage to SP-A and thereby elicit surfactant dysfunction.²⁸ Similar data have been obtained using isolated neutrophils, indicating that surfactant might be important in limiting a secondary tissue damage mediated by neutrophils. This mechanism may be of special relevance in children and adults with acute respiratory failure due to pneumonia. Case reports and smaller clinical studies²⁹ indicate that surfactant replacement might also be helpful in older patients with ARDS triggered by infection,³⁰ and there are several experimental studies reporting improved lung function in animals with experimental bacterial,³¹ protozoal,³² or viral^{33 34} pneumonia treated with surfactant.

In conclusion, there is increasing evidence that apart from its well characterised biophysical properties, surfactant is of major importance in the lung defence system¹³ in both viral and bacterial infections. In our study we observed improved lung function and mitigation of bacterial growth following surfactant treatment in experimental neonatal GBS pneumonia in a preterm rabbit model. The present animal model should be useful for studies aiming at optimising strategies for treatment of congenital pneumonia, especially dose and timing of surfactant administration and efficacy and safety of new recombinant surfactant preparations.

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