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INACTIVATION OF PULMONARY SURFACTANT AND ITS PREVENTION

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Cover: "Surfactant, meconium and dextran"
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Stufen

Wie jede Blüte welkt und jede Jugend
Dem Alter weicht, blüht jede Lebensstufe,
Blüht jede Weisheit auch und jede Tugend
Zu ihrer Zeit und darf nicht ewig dauern.
Es muß das Herz bei jedem Lebensrufe
Bereit zum Abschied sein und Neubeginne,
Um sich in Tapferkeit und ohne Trauern
In andre, neue Bindungen zu geben.
Und jedem Anfang wohnt ein Zauber inne,
Der uns beschützt und der uns hilft, zu leben.

Wir sollen heiter Raum um Raum durchschreiten,
An keinem wie an einer Heimat hängen,
Der Weltgeist will nicht fesseln uns und engen,
Er will uns Stuf' um Stufe heben, weiten.
Kaum sind wir heimisch einem Lebenskreise
Und traulich eingewohnt, so droht Erschlaffen,
Nur wer bereit zu Aufbruch ist und Reise,
Mag lähmender Gewöhnung sich entraffen.

Es wird vielleicht auch noch die Todesstunde
Uns neuen Räumen jung entgegen senden,
Des Lebens Ruf an uns wird niemals enden...
Wohlan denn, Herz, nimm Abschied und gesunde!

(Hermann Hesse)

Stages

As every flower fades and as all youth
Departs, so life at every stage,
So every virtue, so our grasp of truth,
Blooms in its day and may not last forever.
Since life may summon us at every age
Be ready, heart, for parting, new endeavor,
Be ready bravely and without remorse
To find new light that old ties cannot give.
In all beginnings dwells a magic force
For guarding us and helping us to live.

Serenely let us move to distant places
And let no sentiments of home detain us.
The Cosmic Spirit seeks not to restrain us
But lifts us stage by stage to wider spaces.
If we accept a home of our own making,
Familiar habit makes for indolence.
We must prepare for parting and leave-taking
Or else remain the slaves of permanence.

Even the hour of our death may send
Us speeding on to fresh and newer spaces,
And life may summon us to newer races.
So be it, heart: bid farewell without end.

To my lovely family, Silke, Kim Joana, Tjark Elija and Erik Joel

ABSTRACT

Pulmonary surfactant is a lipoprotein complex coating the conducting airways down to the terminal airspaces. Its main function is to lower surface tension at the air liquid interface thus preventing alveolar collapse at end expiration. Primary surfactant deficiency is the main cause of neonatal respiratory distress syndrome (RDS) and treatment with exogenous pulmonary surfactant improves the course of the disease significantly. Furthermore, secondary surfactant deficiency caused by inactivation of endogenous surfactant occurs in older patients with acute RDS (ARDS), pneumonia or meconium aspiration syndrome (MAS). Abnormal alveolar presence of substances like aspirated meconium or leaking serum proteins inhibits surface activity. This inactivation of endogenous surfactant can be overcome by increasing the surfactant concentration with exogenous surfactant. In the clinical setting modified natural surfactants extracted from animal lungs are used most commonly. However, a new generation of surfactant preparations containing synthetic proteins are currently under development to improve supply and to lower costs. In our project we investigated the sensitivity of exogenous surfactant preparations to different types of inactivation and tried to find ways for improving their resistance. *In vitro* we found new synthetic surfactant containing proteins to be more resistant to meconium inactivation than modified natural surfactants. Meconium induced inactivation of modified natural surfactant was reversible, especially in the presence of calcium or polymyxin B (PxB), a cross linking protein of phospholipid films with antimicrobial properties. PxB enriched surfactant also preserves antibiotic characteristics *in vitro* and in an animal model of neonatal pneumonia. Accidentally, we observed surfactant inactivation by silicone oil dissolved from syringes that are in clinical use. This type of inactivation may lead to significant deterioration of lungs in an animal model of RDS. By comparison of surfactant preparations comprising the hydrophobic surfactant protein B and/or C we pointed out that both proteins are needed for alveolar stability.

LIST OF PUBLICATIONS

I. Herting E, Rauprich P, Stichtenoth G, Walter G, Johansson J, Robertson B. Resistance of different surfactant preparations to inactivation by meconium. Pediatric Research 2001, 50: 44-49.

II. Herting E, Stichtenoth G, Robertson B. Syringes with rubber-coated plungers and inactivation of surfactant. Lancet 2003, 361: 311-313.

III. Stichtenoth G, Jung P, Walter G, Johansson J, Robertson B, Curstedt T, Herting E. Polymyxin B/pulmonary surfactant mixtures have increased resistance to inactivation by meconium and reduce growth of Gram-negative bacteria *in vitro*. Pediatric Research 2006, 59, 407-411.

IV. Almlén A, Stichtenoth G, Linderholm B, Hägerstrand Björkman M, Robertson B, Johansson J, Curstedt T. Surfactant proteins B and C are both necessary for alveolar stability at end expiration in premature rabbits with respiratory distress syndrome. J Appl Physiol 2008, 104, 1101-1108.

V. Stichtenoth G, Linderholm B, Almlén A, Hägerstrand Björkman M, Byman I, Nordling K, Robertson B, Johansson J, Herting E, Curstedt T. Inactivation of pulmonary surfactant by silicone oil *in vitro* and in ventilated immature rabbits. Critical Care Medicine 2009; 37: 1750-1756.

VI. Stichtenoth G, Linderholm B, Hägerstrand Björkman M, Walter G, Curstedt T, Herting E. Prophylactic intratracheal administration of polymyxin B /surfactant mixtures prevents bacterial growth and maintains compliance in E.coli pneumonia of newborn rabbits. Pediatric Research, submitted.

CONTENTS

LIST OF ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
CBS	Captive bubble surfactometer
CFU	Colony forming unit
DPPC	1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine
ECG	Electrocardiogram
GBS	*Group-B streptococci*
LGV	Lung gas volume
LPS	Lipopolysaccharide, endotoxin
MAS	Meconium aspiration syndrome
PBS	Pulsating bubble surfactometer
PDMS	Polydimethylsiloxane
PEEP	Positive end expiratory pressure
PL	Phospholipid
PLPE	1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phosphoglycerol
PxB	Polymyxin B
RDS	Respiratory distress syndrome
rSP-C	Recombinant SP-C
SO	Silicone oil
SP	Surfactant associated protein
T_I	Time to inflate 66% of tidal volume
VT	Tidal Volume
γ	Surface tension

PREFACE

I came to the laboratory for surfactant research at the University of Göttingen as a medical student working part time as a paramedic for providing my studies. I was fascinated by paediatric and neonatal intensive care. The interpersonal relation to people surrounding me had always a significant impact on whether I felt comfortable in a team or not. Taken together these requirements with my enthusiasm for Scandinavia, especially Sweden, it was an accidental luck being forwarded to Egbert Herting when searching for an experimental research project. I soon get technically fascinated by surface tension measurements and planning my projects. Soon, the pulsating bubble surfactometer as the central method of my little project became refined by optimising the data analysis. When Egbert paved the way to the surfactant laboratory in Stockholm I was still a student. There, I was received by a friendly, well organised international research team. Though Curosurf showed the worst resistance to inactivation by meconium, my studies were nevertheless accepted by the surfactant experts Bengt Robertson and Tore Curstedt. They let me dive deeper into the field and enabled participation on scientific meetings like the Mini Nobel Symposium 1999, which was organised by Bengt. There I had a short conversation with Peter Agre, who I saw the next time in the German TV after receiving the Nobel Prize in 2003.

When starting my job as a physician in Göttingen, I was even more fascinated by learning the practical skills in neonatology, seeing these small neonates surviving by means of exogenous surfactant. The blessing of surfactant therapy met my fate when our son Tjark Elija was born prematurely soon developing respiratory distress. Retrospectively, I am even more grateful of getting the opportunity for full time research at the Karolinska Institutet. It was a very exciting period and one of the important things I realised was that it is an advantage, as a research fellow, to have a clinical background. Unfortunately, due to lack of time, it is nearly impossible doing both good clinical work and good research. As a summary of the lucky times in full time research, I enjoyed writing this book. Have fun in reading it!

Guido Stichtenoth, Lübeck in May 2009

1 INTRODUCTION

1.1 HISTORY

The word surfactant is an acronym which describes a **sur**face **act**ive **age**nt. Pulmonary surfactant coats the conducting airways down to the terminal airspaces. Surfactant was first described in 1929, by suggesting the presence of a surface active film and its relevance to the newborn's first breath (1). 25 years later stable microbubbles were described in a fluid, which is squeezed out of lungs and their impact on alveolar stability during expiration was presumed (2). Then in 1959 Avery and Mead suggested that preterm neonates dying of hyaline membrane disease (respiratory distress syndrome, RDS) had a deficiency of pulmonary surfactant (3). Pattle and Thomas claimed that the surface-acitve material was a lecithin-protein complex (4). Based upon this finding a surfactant preparation of lecithin was developed and administrated as aerosol, but this approach failed (5). The effectiveness of natural surfactant derived from animal lungs was demonstrated in isolated lungs by Rüfer (6) and by Robertson and Enhörning in newborn premature rabbits (7). The first report of a successful treatment of RDS with bovine surfactant was published 1980 by Fujiwara (8). Since that time treatment of RDS with surfactant has developed into a save and effective procedure contributing to a significant decrease of morbidity and mortality in premature neonates (9). Surfactant replacement therapy has been established in meconium aspiration syndrome and neonatal *Group-B streptococcal* pneumonia (10). Moreover, treatment with exogenous surfactant has shown benefits in acute respiratory distress (ARDS) or acute lung injury (11, 12).

1.2 COMPOSITION OF NATURAL SURFACTANT

Surfactant consists mainly of phospolipids and surfactant specific proteins. However, it also contains neutral lipids (e.g. free fatty acids, cholesterol). Natural surfactant has a complex composition with at least fifty different phospholipids (PLs) (13) of which phosphatidylcholines account for 70-80%. The predominant lipid component, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), is responsible for reducing surface tension to low values during expiration. The lipid composition of pulmonary surfactant differs between mammalian species and may depend on the respiratory rate (14). Complete natural lavage surfactant contains up to 10% of specific surfactant proteins (SP-), SP-A, SP-B, SP-C, and SP-D (15). SP-A represents approximately 5% of whole surfactant and 50% (by weight) of the total protein content (16). The SPs can be

divided into hydrophilic (SP-A and –D) and hydrophobic (SP-B and –C) proteins. SP-A and -D belong to the protein family of collectins (17). SP-A is assembled from 18 monomeric subunits (6 trimers) of which each monomer has a molecular weight of 28-36 kDa (18). SP-D like SP-A is an oligomer consisting of 12 monomer (4 assembled trimers) at a molecular weight of 43 kDa. SP-B is a homodimeric molecule with a weight of 17 kDa. It may interact with the surface of the lipid bilayers by means of four or five amphipatic α-helices in each monomer (19). Together with SP-C, which is an even smaller peptide of 4 kDa encoded on chromosome 8, it is synthesised in alveolar type II cells into intracellular densly packed phospholipid layers called the lamellar bodies. SP-C contains thioester-linked palmitoyl groups in the N-terminal part (20), an α-helical part of the molecule is inserted in the lipid bilayer and orientated near parallel to the phospholipid acyl groups (21). SP-B and SP-C constitute ~1.3 % and ~4 % (w/w), respectively, of natural surfactant (22).

1.3 PHYSIOLOGY

The main function of pulmonary surfactant is the reduction of surface tension at the air-liquid interface. Moreover, surfactant is the airway-barrier between the environment and the human body. Thus it has a role in host defence, fluid balance and gas exchange. Via exocytosis of lamellar bodies, surfactant is secreted into the hypophase of the alveoli. Here, surfactant forms a lattice-like structure, which is called tubular myelin and can be visualised with electron microscopic techniques. Tubular myelin is an intermediate form, consisting of phopholipids, SP-A, -B and –C (23). SP-A is found in the corners of the lattice structure. The final faith of surfactant is to reach the air liquid interface.

SP-B and SP-C are crucial for the adsorption of the surfactant film at the alveolar air-liquid interface (24, 25), whereas the hydrophilic proteins SP-A and SP-D are mainly involved in innate host defence (26) (Table 1). The SPs orchestrate with the phospholipid film resulting in a reduction of surface tension, which during expiration achieves surface tensions close to 0 mN/m. It is believed that squeezing out of non DPPC components contributes to this mechanism (27, 28, 29). Both SP-B and SP-C knockout mice are unable to establish normal lung function (30, 31). Infants with SP-B deficiency also have abnormal SP-C and develop progressive, lethal respiratory failure in the neonatal period while patients with SP-C deficiency will develop progressive lung fibrosis (32). Despite their great impact on adsorption of phospholipids at the air-

liquid interface, the exact mechanisms of action of SP-B and SP-C are still incompletely understood.

Table 1: Properties of surfactant proteins (SP-);

SP-A	SP-D	SP-B	SP-C
hydrophilic		hydrophobic	
innate host defense		formation, refinement, reduction of surface tension	
regulation		essential*	
recycling		alveolar stability	

* neonates with normal SP-A, no SP-B and abnormal SP-C (proform) show lethal RDS (33)

Surfactant is either recycled by reuptake of type II cells or degraded by alveolar macrophages. *In vitro* samples of natural surfactant can be separated into active lamellar like body large aggregate forms and less active small aggregates (34).
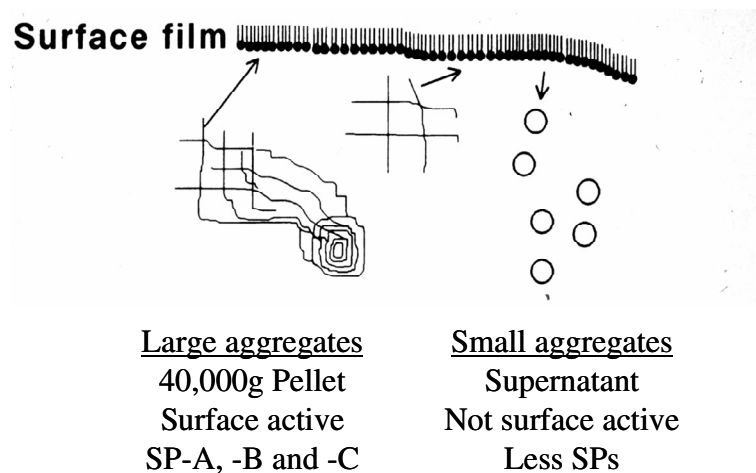

Large aggregates   Small aggregates
40,000g Pellet   Supernatant
Surface active   Not surface active
SP-A, -B and -C   Less SPs

Figure 1: Conversion of surface active large aggregates to non-surface active small aggregates. Modified from (35).

1.4   PRIMARY SURFACTANT DEFICIENCY

Respiratory distress. Deficiency of pulmonary surfactant is the underlying cause of the respiratory distress syndrome (RDS) in premature neonates, and the intratracheal administration of surfactant, purified from animal lungs, greatly reduces morbidity and mortality (36, 37). Due to prematurity of the lungs, the alveolar cells have not differentiated sufficiently into type I and type II cells. Thus, adequate synthesis of

surfactant for postnatal life has not started yet. End-expiratory collapse of the alveoli and bronchioli, atelectasis, shear forces that lead to airway epithelial damage, capillary leak and hypoxia contribute to the pathology of the disease finally resulting in formation of hyaline membranes (38).

1.5   SECONDARY SURFACTANT DEFICIENCY

Secondary surfactant deficiency caused by inactivation of the surfactant system may occur in patients with mature lungs. This inactivation describes a loss or reduction of the physical properties of surfactant and is consequently different to primary deficiency. It might be caused by interference with delivering surfactant to the surfactant monolayer or with refinement during film compression. Substances negatively affecting the surface activity might be present in the alveolar space (39). Those originate from aspiration via the conducting airways or from the blood (Table 2).

Table 2:   Substances capable for inactivation of surfactant
(40, 41, 42, 43, 44, 45, 46, 57).

- plasma proteins (fibrinogen, albumin)
- haemoglobin, bilirubin
- proteases/lipases
- oxidants
- lipids/oils (lamp oil)
- gastric contents
- meconium
- water (drowning)

General clinical conditions promoting surfactant inactivation might be accompanied by aspiration, inhalation, inflammation, ventilator trauma or shock. Hence, surfactant inhibitors may reach the alveolar space and plasma proteins such as albumin or fibrinogen may leak into the airways as a consequence of increased vascular permeability caused by endothelial and epithelial dysfunction, interstitial inflammation and oedema, for example (47, 48). This may lead to ARDS (acute (adult) RDS). Suggested mechanisms interfering with surface activity in ARDS may be competition between surfactant and lipid substances or proteins for air-liquid interface (49; 50), binding or aggregation of surfactant components, enzymatic degradation (51), dilution

and removal due to pulmonary oedema (39) and clogging the alveolar spaces with debris. In addition, alveolar type II cells might be injured. As a result of the described effects surfactant is inactivated and pulmonary gas exchange is reduced (Figure 2). To treat ARDS, relatively large doses of surfactant are required to overcome the amount of surfactant inhibitors present in the airways (52). Thus, surfactant preparations used for treatment of ARDS should be relatively resistant to inactivation (53). Recent advances in the synthesis and heterologous expression of lung SPs or their analogues might allow the production of designer surfactants (54) that are highly resistant to surfactant inhibitors.
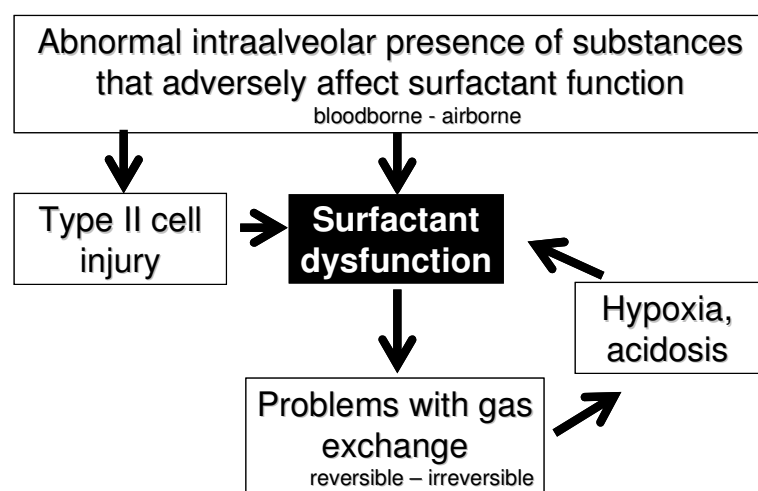

Figure 2: Pathway of surfactant inactivation.

1.5.1 Meconium aspiration

A disease similar to ARDS may occur in neonates after aspiration of meconium, which can result in severe respiratory failure in term neonates (55, 56). Surfactant inactivation is believed to play a key role in the pathophysiology of the meconium aspiration syndrome (MAS), and direct inhibition of the surface tension-lowering activity of surfactant by meconium has been demonstrated *in vitro* (57, 58). In addition, *in utero* events, such as hypoxia, that trigger meconium passage into the amniotic fluid may interfere with surfactant synthesis and secretion by alveolar type II cells (38). In MAS, an early phase of airway obstruction and surfactant dysfunction is observed, which is followed by a second phase with a marked inflammatory response in the airways (59). The liberation of cytokines, oxygen radicals, enzymes (*e.g.* neutrophil elastase) from phagocytic cells, and, in some cases, secondary bacterial pneumonia may enhance surfactant inactivation. Apart from its biophysical effects at the air-liquid interface, the

immunomodulatory functions (26) and the contribution to mucociliary clearance of aspirated particles may be disturbed in MAS. This may result in hypoxia and acidosis, which further reduces surfactant activity and induces pulmonary vasoconstriction (Figure 3).
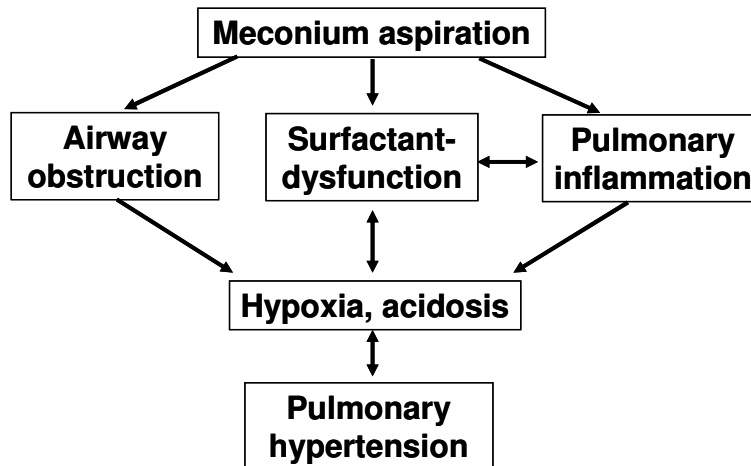

Figure 3: Pathophysiology of meconium aspiration syndrome.

Animal models (60, 61) and clinical studies (62) demonstrate that lung function and oxygenation in MAS can be improved by instillation of large doses of modified natural surfactants containing phospholipids, SP-B and SP-C.

1.5.2 Neonatal pneumonia

Gram-positive bacteria like *Group-B streptococci* (GBS) or *Staphylococcus aureus* and Gram-negative microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae*, or *Enterobacter cloacae* are common pathogens causing serious neonatal infections, including pneumonia and sepsis (63, 64, 65). The infection leads to pulmonary inflammation with accumulation of cytokines in the lung, leakage of plasma proteins into the alveoli, and surfactant inactivation. In experimental neonatal GBS pneumonia in rabbits, treatment with modified natural surfactant significantly reduced bacterial growth compared to control animals not receiving surfactant (66). Clinically, treatment of neonatal pneumonia includes endotracheal surfactant administration and antibiotic therapy (10).

2 AIMS OF THE THESIS

2.1 GENERAL AIM

The recurrent theme of this investigation is:

How can we improve the function of pulmonary surfactant and how can we prevent surfactant dysfunction?

Since surfactant inactivation occurs by different pathways, there are different therapeutic strategies, which subsequently require different methods of investigation.

2.2 SPECIFIC AIMS

- To compare different commercially available surfactant preparations for their relative resistance to inactivation by meconium.

- To investigate effects of mixtures, consisting of surfactant, the antimicrobial peptide polymyxin B and/or calcium chloride ($CaCl_2$), on meconium-induced decrease in surface activity and on bacterial growth.

- To study the effects of mixtures of surfactant/polymyxin B in an animal model of neonatal pneumonia.

- To investigate why surface activity of surfactant is reduced after contact with one-mL three part syringes.

- To study effects of silicone oil used for lubrication of syringes on surface activity *in vivo* and *in vitro*.

- To evaluate the effects of surfactant preparations containing SP-B and/or SP-C on alveolar stability in immature neonatal rabbits.

3 MATERIAL AND METHODS

3.1 SURFACTANT

3.1.1. Natural surfactant

Full natural surfactant containing all SPs was obtained by bronchoalveolar lavage of adult rabbit lungs. After cell debris was removed by centrifugation at 500g, the supernatant was centrifuged at 6000g at 4°C for 1h and the surface-active material was harvested from the pellet. Phospholipid concentration was determined using Bartletts method (67). For comparison of the composition of natural surfactant and the exogenous surfactant preparations described below see Figure 4.

3.1.2. Modified natural surfactant preparations

Common components in these preparations are phospholipids, SP-B and SP-C: Curosurf (Chiesi Farmaceutici, Parma, Italy) is produced from minced pig lungs and consists of 99% phospholipids and ~0.7 % and ~0.6 % (w/w) of the hydrophobic surfactant proteins (SP-B and SP-C), respectively (68). Alveofact (Dr. Karl Thomae Ltd, Biberach, Germany), a compound obtained from bovine lung lavage, is composed of 90% phospholipids, approximately 1% hydrophobic proteins, 3% cholesterol, 0.5% free fatty acids, and other components such as triglycerides. Survanta (Abbott Ltd, Wiesbaden, Germany) is prepared by lipid extraction of minced bovine lungs and contains approximately 84% phospholipids, 1% hydrophobic proteins, and 6% free fatty acids. DPPC is added in this preparation together with palmitic acid and tripalmitin to standardise the composition.

3.1.3. Protein-free synthetic surfactant preparations

Exosurf (Wellcome, Burgwedel, Germany), a synthetic, protein-free surfactant, is composed of approximately 85% DPPC mixed with cetyl alcohol and tyloxapol. Pumactant (ALEC (artificial lung-expanding compound), Britannia Pharmaceuticals, Redhill, Surrey, UK) is an artificial, protein-free preparation composed of DPPC and phosphatidylglycerol at a weight ratio of 7:3. Protein free synthetic surfactants are no longer in clinical use, since it has been shown that these agents are inferior to surfactant derived from natural sources (69).

3.1.4. Synthetic surfactant preparations containing peptides or recombinant proteins

A mixture of phospholipids and the hydrophic proteins SP-B and SP-C was isolated from porcine lungs as described previously (25), whereupon the proteins were separated from the phospholipids using liquid gel chromatography in a Sephadex LH-20® column (70). Finally, SP-B was separated from SP-C by size-exclusion chromatography on a Sephadex LH-60® column (25). The content of SP-B and SP-C was determined using amino acid analysis (68). SP-C33 is an SP-C analogue and was provided by Chiesi Farmaceutici S.p.A. (71, 72). Shortly, this peptide differs from human SP-C by removal of the two N-terminal amino acid residues, replacement of the palmitoyl-Cys residues with Ser, Leu with Lys in position 12 and the poly-Val part with a poly-Leu sequence (71, 72). Premature newborn rabbits with surfactant deficiency, treated with synthetic surfactant containing 2% of SP-C33 in DPPC: 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phosphoglycerol (POPG) (68:31, w/w) and ventilated with a standardised sequence of pressure without positive end expiratory pressure (PEEP), have similar tidal volumes compared to those treated with a modified porcine surfactant preparation (Curosurf). However, lung gas volumes, reflecting the functional residual capacity, are much lower in animals treated with the synthetic surfactant (71). The phospholipids 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), DPPC and POPG were provided by Chiesi Farmaceutici (Parma, Italy). 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE) was supplied by Sigma-Aldrich Sweden AB (Stockholm). DPPC/PLPC/POPG/PLPE (55:25:15:5, by weight) or DPPC/POPG (68:31, w/w) dissolved in chloroform/methanol (1:1, v/v) were mixed with peptides in the same solvent. The peptide contents were 2% SP-C33, 4% SP-C33, 2% SP-C33+2% SP-B, 2% SP-B or 2% SP-C. The mixtures were evaporated and resuspended to a final phospholipid concentration of 80 mg/mL.

Cycling studies: Two mL of these surfactant preparation, diluted with saline to 10 mg/mL, were added to a 15 mL (10 x 1.4 cm) capped glass tube that was rotated at 20 rpm for 7 days at 37°C in order to obtain a maximal change of the surface area. Aliquots were taken from the samples at time 0, and after 1, 3 and 7 days of cycling to measure surface activity.

Polymyxins are peptides derived from *Bacillus polymyxae* with antimicrobial activity against a wide spectrum of Gram-negative bacteria including those considered to be multi-resistant, e.g. *Pseudomonas aeruginosa*. Pharmacologically, they are used as topical antibiotics and can be administered to lungs by inhalation. They are rarely used systemically since adverse neurotoxic and nephrotoxic effects have been described (73, 74). PxB like SP-B may cross-link two juxtaposed phospholipid bilayers, and addition of SP-B or PxB to synthetic pulmonary surfactant improved surface activity (75). For Paper III and VI PxB (Sigma Aldrich; >6000 Units/mg) was suspended at 80, 40 and 20 mg/mL in saline and mixed with modified porcine surfactant 80 mg/mL (Curosurf; Chiesi Farmaceutici, Parma, Italy) at a ratio 1:100, thus giving preparations of Curosurf/PxB 1%, 0.5% and 0.25% (w/w).

A 21-residue leucine/lysine peptide (KL_4 = Sinapultide), was synthesised and combined with DPPC and phosphatidylglycerol at a weight ratio of 3:1 and palmitic acid (15%) in an isotonic aqueous suspension. The KL_4 peptide, added to the lipids at a concentration of 3%, is suggested to mimic the C-terminal part of SP-B (76, 77). However, in contrast to KL_4, the C-terminal part of SP-B has an amphipathic surface, i.e. positive residues are situated on one face while the mostly unpolar residues are situated on the opposite face (78, 79). KL_4 has been tested *in vitro*, in animal experiments, and in clinical trials for treatment of RDS, MAS, and ARDS (80, 81). Larger trials with a commercial product (Surfaxin, Discovery Laboratories, Doylestown, PA, U.S.A.) showed that the preparation is safe and almost as efficient as animal derived modified natural surfactants (82). Nevertheless, there are concerns and precautions with respect to the design of the clinical studies (non-inferiority testing), bias due to sponsoring, the dosing and the handling of Surfaxin, which forms a gel and needs to be heated to 44°C before use (83).

Recombinant SP-C (rSP-C) surfactant (kind gift from D. Häfner, Byk Gulden, Konstanz, Germany) is prepared as an amorphous dry powder and is resuspended in 0.9% NaCl. The final suspension contains (by weight) 1.8% rSP-C, 63% DPPC, 28% phosphatidylglycerol, 4.5% palmitic acid, and 2.5% $CaCl_2$. *In vitro* studies and animal experiments indicate excellent biophysical function and improved gas exchange (84). On the other hand, a large clinical trial enrolling ARDS patients in Europe, South Africa, the United States, and Canada (85) with a commercial product (Venticute, Nycomed, Konstanz, Germany) was recently stopped due to lack of effectiveness. A post hoc subgroup analysis of patients with severe respiratory insufficiency due to

pneumonia or aspiration showed besides improved oxygenation a reduced mortality (86).
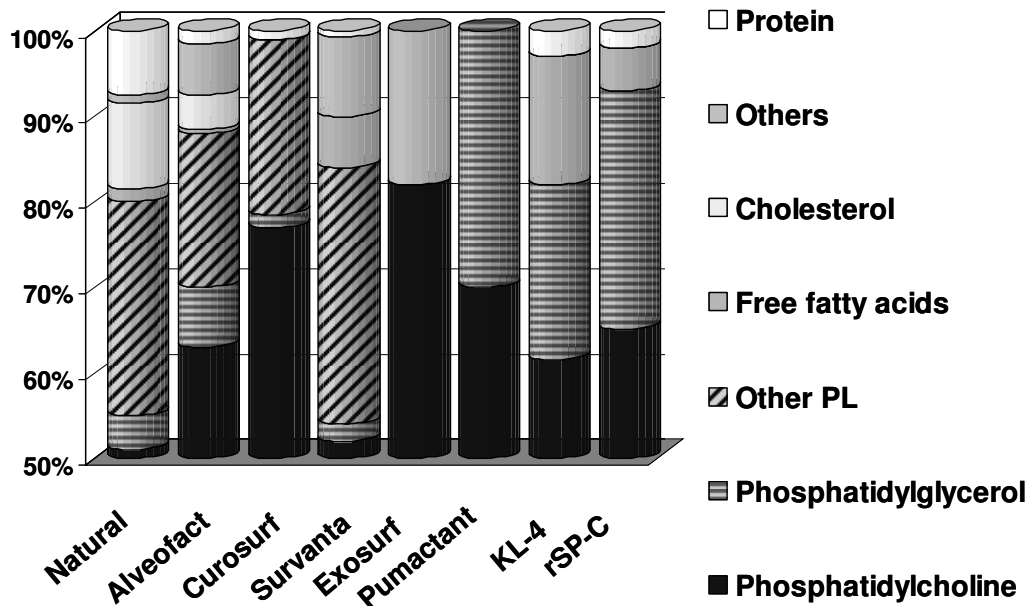

Figure 4: Composition of different surfactant preparations. Modified from Wauer (87) and Seeger et. al. (88). All preparations contain >70% phospholipids.

3.2. SAMPLE PREPARATION

In vivo the concentration of the used surfactants was 80 mg/mL. In most of our *in vitro* studies the concentration of surfactant was 2.5 mg/mL, which is close to the estimated critical concentration of surfactant in fetal lung liquid at birth (89). For comparison, concentrations of 5 or 10 mg/mL were used. The surfactant preparations were diluted in saline and in some studies in saline containing 1.5 mmol/L $CaCl_2$.

3.3. MECONIUM

The first meconium was collected from 16 infants, lyophilised and pooled (entry criteria: gestational age, 37-41 weeks; birth weight, between 3rd and 97th percentiles; no signs of maternal infection; clear amniotic fluid; no signs of asphyxia (umbilical cord pH >7.25); no bacterial contamination of meconium ($<10^4$ bacteria/mL)). The dry meconium was resuspended under sonication in saline 0.9% or saline with 1.5 mM $CaCl_2$ (90). As up to 200 g of meconium may be passed by a full-term neonate at birth (55) and the volume of amniotic fluid at birth rarely exceeds 2000 mL, meconium concentrations >20 mg/mL can certainly be achieved even assuming uniform distribution.

3.4. SILICONE OIL – SURFACTANT – SAMPLES

Syringes containing rubber plungers are lubricated with silicone oil (SO) and are applied for administration of exogenous pulmonary surfactant in small neonates with RDS. Nearly 70% of total SO can be extracted by rinsing these syringes with water or soybean oil (91). Thus, silicones dissolved from syringes or from other medical devices, e.g. siliconised tubes, might enter the lungs during treatment of the patient. The effects of SO contamination of exogenous surfactant are unknown. SOs mainly consist of linear (L_n; formula: CH_3-$(SiO(CH_3)_2)_n$-$SiO(CH_3)_3$), but also of cyclic (D_n; formula: -$SiO(CH_3)_2)_n$-) chains of Polydimethylsiloxanes (PDMS) with a wide range of molecular masses. PDMS are basically inert (92). One-mL three part syringes, consisting of a cylinder plus a plunger with a rubber seal lubricated with SO, were obtained from B.Braun, Melsungen, Germany.

SO was mixed with Curosurf (80 mg/mL) or DPPC/ POPG 68:31 (w/w), giving ratios of 0-1.3 mg SO/mg phospholipid. Omnifix F syringes were rinsed 3 or 15 times with Curosurf 80 mg/mL and for comparison once with Curosurf diluted to 2.5 mg/mL with saline.

3.5. MASS SPECTROMETRY OF SILICONE SAMPLES

SO was diluted in organic solvents (chloroform/methanol or n-heptane/ethanol). Additionally, after phase separation using chloroform/methanol/water at volume proportions of 8:4:3 (93), the hydrophobic phase was mixed with small amounts of NaCl 0.9%. Syringes were rinsed 10 times with n-heptane or Curosurf, diluted to 2 mg/mL with saline. These samples underwent extraction with n-heptane/water 1:1(v/v) and the isolated hydrophobic phase was evaporated under nitrogen and resuspended in chloroform/methanol 1:1 (v/v).

The samples, which were prepared in acid cleaned and chloroform rinsed glass tubes in order to avoid external silicone contamination, were infused by platinum glass capillaries (Medium NanoES spray capillaries, Proxeon Biosystems, Odense, Denmark) into a quadrupole–time of flight mass spectrometer (Q-Tof 2, Micromass, Manchester, UK). Mass spectra were scanned within a mass/charge (m/z) range of 35-10,000 and compared with spectra of typical PDMS molecules.

3.6. *WESTERN BLOTTING* OF SILICONE SAMPLES

SO/Curosurf preparations (non-extracted) were compared with sub-fractions derived from organic extraction (93). The lower hydrophobic (chloroform/methanol) and upper hydrophilic (methanol/water) phases were evaporated and resuspended in 50 mM Tris,

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50 mM NaCl, pH 7.4. Some samples were desalted to exclude interference during electrophoresis. *Western* blotting was performed using NuPAGE® Novex 12% Bis-Tris SDS gels for electrophoresis, polyvinyldifluoride transfer membranes, anti-SP-C or anti-SP-B primary antibodies (derived from rabbits; kind gift of T.Weaver, Cincinatti, OH, USA) and horseradish peroxidase linked anti-rabbit IgG (Amersham Biosciences, Little Chalfont, UK) as secondary antibodies. See Blue Plus2 and Magic Mark XP (Invitrogen, Carlsbad, CA) were used as a loading control.

3.7. BACTERIA

Escherichia coli (strain ATTC 25922), GBS (strain 090 Ia Colindale, a high-density variant that lacks a polysaccharide capsule, a kind gift from Stellan Håkanson, University of Umeå, Sweden), or *S.aureus* were incubated at 37°C to reach the mid-logarithmic growth phase and bacterial concentrations were adjusted by measuring optical density of the suspensions at 595 nm to transfer approximately 10^8 colony forming units (CFU) to sterile tubes. Then samples were incubated 0-5 h in saline or in meconium in the presence or absence of Curosurf with or without PxB (0.1 mg/mL). Bacterial growth experiments were conducted at final concentrations of 20 mg/mL meconium and 10 mg/mL Curosurf both in saline. Five repeats of each experiment were done. For neonatal *E.coli* pneumonia studies (paper VI), a bacterial stock containing approximately 10^8 *E.coli*/mL was prepared.

3.8. *IN VITRO* MODELS FOR DETERMINATION OF SURFACTANT ACTIVITY

3.8.1. The pulsating bubble surfactometer

The pulsating bubble surfactometer (PBS, Electronetics Corporation, Buffalo, NY, USA), developed by Enhörning (94), can measure biophysical activity in a dynamic system mimicking *in vitro* an alveolus of the lung (Figure 5): In short, a bubble communicating with ambient air is generated in a test chamber filled with surfactant and pulsated by means of a piston system. After an initial static adsorption period, the bubble undergoes cyclic changes in radius between 0.55 mm and 0.40 mm, corresponding to 50% area compression. The pressure changes are recorded and a computer calculates surface tension, using the law of Laplace (Figure 5). Measurements were made at 37°C. Static surface tension was recorded after 2 min of adsorption (γ_{ads}) at 0.4 mm and dynamic surface tensions at minimum bubble size (γ_{min}) and maximum bubble size (γ_{max}) were recorded during 5 min of pulsation at a rate of 20 cycles/min.
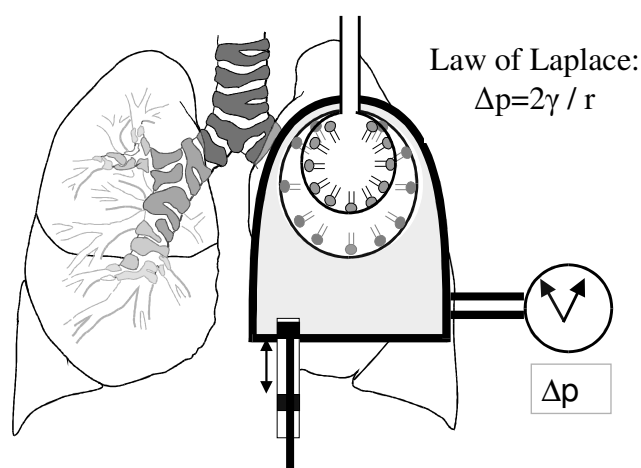
Figure 5: Scheme of the PBS as a model of an alveolus.

3.8.2. The captive bubble surfactometer

The captive bubble surfactometer (CBS) measures biophysical activity of a bubble generated in a sample chamber without communication to ambient air. The surface tension is measured in the CBS by analysis of the bubble shape and size (95, 96). Stepwise, the bubble can be cyclically compressed and expanded (quasi static cycling), using compression rates up to 90% (97). The test chamber was initially filled with 10% sucrose in saline. Approximately 2 µl of surfactant (10 mg/ml) were injected into the sample chamber and allowed to migrate by buoyancy to an agarose ceiling. An air bubble was then placed under the ceiling in contact with the surfactant preparation and surface tension was measured during five minutes of adsorption. The sample chamber was sealed and the quasi static cycling was initiated. The bubble was compressed stepwise until a surface tension less than 5 mN/m was reached or to 50% area compression, and expanded to the initial size thereafter. This manoeuvre was repeated five times.

3.8.3. The Wilhelmy balance

Spreading rates of the surfactant samples were evaluated (Paper III) using a modified Langmuir-Wilhelmy balance (Biegler, Mauerbach, Austria; 98). The trough was filled with saline heated to 37°C. The surface area was 20 cm^2 and each sample was applied as a single droplet on the hypophase 4 cm from the dipping sensor plate. The resulting change in surface tension was recorded during 5 min after application of the test material. Each applied sample contained the amount required to coat the surface with two monolayers of saturated phospholipids, calculated with a hypothetical area of approximately 40 Å^2 per saturated phosphatidylcholine molecule (99).

3.9. ANIMAL MODELS

3.9.1. The immature newborn rabbit model

Preterm newborn rabbits obtained at a gestational age of 27 days (term 31 days) were tracheotomised at birth and randomly stratified to different treatment groups. The animals were kept in pletysmograph boxes at 37°C and ventilated in parallel with 100% oxygen at a frequency of 40/min and an inspiration/expiration ratio 1:1. To open up the lungs, peak inspiratory pressure was first set to 35 cmH$_2$O for 1 min. Then pressure was lowered to 25 cmH$_2$O for 15 min and further on to 20 cmH$_2$O for 5 min and 15 cmH$_2$O for 5 min. Finally, pressure was raised again to 25 cmH$_2$O for 5 min (13, 25, 100), after which the lungs were ventilated for additional 5 min with nitrogen. The experiments were performed without PEEP. Tidal volumes (*VT*) were recorded every five minutes. At the end of the experiment the tracheal cannula was clamped at end expiration, animals were killed, the trachea was ligated, and the lungs were excised and weighed.

Determination of Lung Gas Volumes (*LGV*): This method was developed from previous studies (101, 71). The *LGV*, an equivalent to the functional residual capacity, was determined by the difference between the organovolume of the lung (V_{lung}) and the tissue volume (*V*(tis)). V_{lung} was derived by weighing the volume of water displaced by the submersed lung using the principle of Archimedes (102). *V*(tis) was derived by converting the wet-weight of the lungs into a volume. Therefore, we used excised non ventilated lungs of 32 newborn rabbits, gestational age 27 days, weighed them and determined their *V*(tis), again using the Archimedean principle.
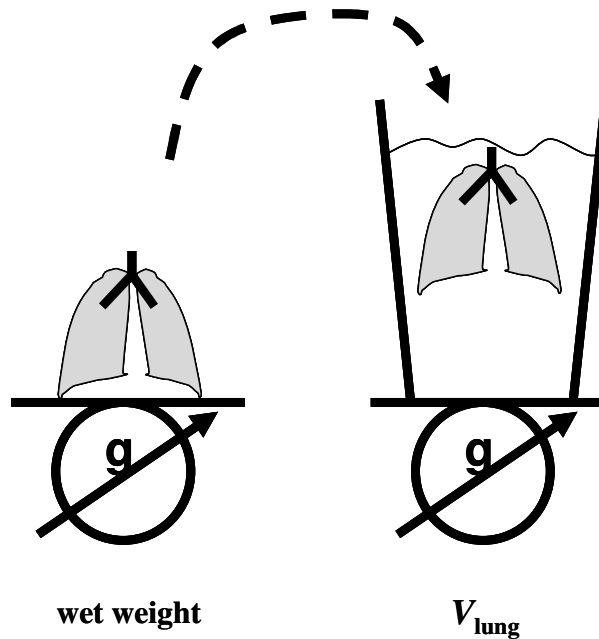

wet weight V_{lung}

Figure 6: Determination of the wet weight and the V_{lung} of the lungs.

We found a linear regression (r^2=0.97; p<0.0001) between wet weight and V(tis) with a wet weight/V(tis) ratio of 1.077 ±0.03 (mean ±SD). The resulting equation for calculation of *LGV* is:

$$LGV[ml/kg] = \frac{V_{lung}[ml] - \dfrac{wet\ weight\ [g]}{1.077}}{body\ weight\ [kg]}$$

Finally the lungs were prepared for histological analysis (see next chapter).

3.9.2. The neonatal pneumonia model

Term *New Zealand White* neonatal rabbits at a gestational age of 29 days were obtained by hysterotomy, anaesthetised and tracheotomised and treated with surfactant and/or PxB preparations. Relaxation was induced and up to 10 animals were ventilated in parallel for 4 h (FiO$_2$=1.0) at a frequency of 40 inspirations/min and an inspiration/expiration-ratio of 0.33. Initially, the lungs were opened with 5 inspirations at a peak insufflation pressure of 35 cmH$_2$O, then a standardised *VT* of 6-7 mL was adjusted. *VT*, peak insufflation pressures and electrocardiograms (ECGs) were recorded at 10 min and 30 min and then at 30 min intervals. After the 15 min recording, animals were inoculated with 5 mL/kg of the *E.coli* stock intratracheally, and the lung opening manoeuvre was repeated. After 4 h, tracheal cannulas were clamped at end expiration. Animals were killed with intracranial lidocaine 2 % (0.5 mL) and abdomens were opened to inspect diaphragms for evidence of pneumothorax. Then the diaphragm was opened and lungs were carefully excised. The right lung was used for determination of bacterial load and the left lung was processed for histological evaluation. Additionally, in some animals the left kidney was excised, prior to opening of the diaphragm.

Determination of bacterial growth: Lungs as well as the left kidneys were weighed. Saline was added to normalise weight to 1 g. Then the samples were homogenised using an OMNI/TH-homogeniser (Omni international, Marietta, GA, USA) with sterile tips. After serial dilution in saline, 100 μl aliquots were transferred to *Petri*-dishes and mixed with warm Columbia agar base (Oxoid CM 331-550 g) containing 5 % (v/v) defibrinated sheep-blood. Dishes were incubated for 24 h at 37°C and CFU/dish were counted. Either the *Petri*-dishes of the homogenate, when CFU count was 0-250 or the dishes of the diluted homogenate next to the homogenate showing 10-250 CFU were considered for evaluation (average count from duplicate dilutions).

Histology: The lungs were fixed in 4% neutral formalin, dehydrated and embedded in paraffin. Transverse sections from the lower lobes, stained with haematoxylin and eosin, were examined by light microscopy (71). The proportion of well aerated alveoli was estimated and classified semi-quantitatively according to a five-grade scale (0: 0, 1: 1-25, 2: 26-50, 3: 51-75, 4: >75%) while a four-grade score was used for estimation of airway epithelial necrosis (0 = absent, 1 = mild, 2 = moderate, 3 = prominent) and, in paper VI, the grade of inflammation (absent, mild moderate, prominent, severe tissue destruction). In addition (paper III), alveolar volume density was measured with a computer-aided image analyser using total parenchyma as reference volume (103).

3.10. DATA ANALYSIS AND STATISTICS

Lung compliance of animals was calculated by dividing *VT* by peak insufflation pressure. Inspiratory time constants (T_I), defined as the period for inflation of 66% *VT*, were calculated from plethysmographic *VT* recordings. Survival of animals was considered when ECG showed a heart rate of >60/min. All data represent the mean ±SD of repeated experiments. Statistical differences were evaluated by ANOVA and Dunnett's or Bonferroni's multiple comparison tests using GraphPad software (Graph Pad Inc., San Diego, CA, USA). Non parametric data were analysed by Kruskal-Wallis test, followed by Dunn's post test. Statistical significance was accepted at $p < 0.05$.

3.11. ETHICAL APPROVAL

The first meconium of healthy term newborn infants was collected with parental consent. The project was approved by the institutional review board of the Medical University of Göttingen. Animal experiments were approved by the local ethical committee for animal research, Stockholms Norra Djurförsöksetiska Nämnd (205/04 and 316/06).

4. RESULTS AND DISCUSSION

4.1. RESISTANCE OF DIFFERENT SURFACTANTS TO INACTIVATION BY MECONIUM (PAPER I)

At a phospholipid concentration of 2.5 mg/mL, all surfactants containing proteins or peptides reduced γ_{min}, determined with a PBS, to <10 mN/m in the absence of meconium, indicating a good biophysical activity. γ_{min} was increased to >10 mN/m at meconium concentrations ≥0.04 mg/mL for Curosurf, Alveofact or Survanta. KL_4 and rSP-C surfactant were more resistant to the inhibitory effects of meconium. An increase of γ_{min} >10 mN/m was observed for rSP-C surfactant at concentrations ≥0.32 mg/mL and for KL_4 at meconium concentrations ≥1.3 mg/mL. Addition of 20 mg/mL meconium was needed to induce a significant increase of γ_{min} for natural rabbit surfactant (Figure 7). The protein-free synthetic surfactants Exosurf and Pumactant did not reach γ_{min} <10 mN/m even in the absence of meconium. Meconium had a dose-dependent inhibitory effect on all examined surfactant preparations.
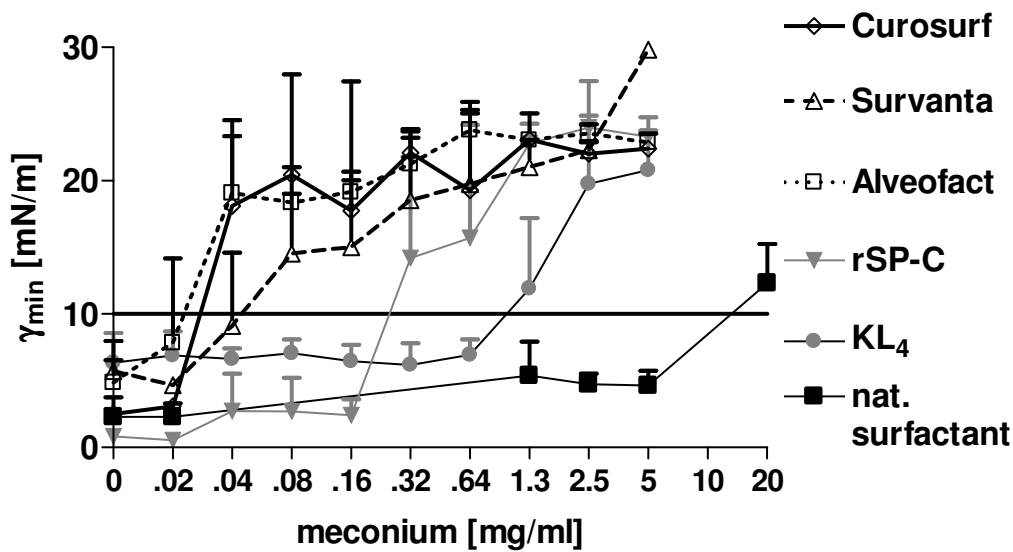

Figure 7: Dose dependent effect of meconium on γ_{min} of different surfactant preparations. Results are expressed as mean ±SD of n=6 repeated measurements. A significant increase in γ_{min} (p<0.01 vs. surfactant without meconium, i.e. 0 mg/mL) occurred at meconium concentrations ≥0.02 mg/mL for Alveofact, at ≥0.04 mg/mL for Curosurf and Survanta, ≥0.32 mg/mL for rSP-C surfactant and at ≥1.3 mg/mL for KL_4 surfactant (One-way ANOVA followed by Dunnett's post test).

The inhibition observed by meconium was reversible. When surfactant concentration was increased to 7.5 mg/mL in inhibited samples, that showed surface tension of >20 mN/m, mean γ_{min} was reduced again to <5 mN/m for Curosurf, Alveofact and rSP-C, and to <15 mN/m for Survanta and KL$_4$.

Meconium is a potent inhibitor as an increase in γ_{min} >10 mN/m was observed for all modified natural surfactant preparations already at a meconium/surfactant ratio of about 1:100. A concentration of 2.5 mg/mL makes surfactant more sensitive to inactivation than concentrations $\geq$5 mg/mL used by other investigators (58). Meconium is a complex mixture containing proteins, cell debris, bile acids, haemoglobin, and bilirubin metabolites, for example (55). All of these components are capable of inhibiting surfactant function (104). Electron microscopic studies noticed that meconium resulted in a transformation of surfactant from dense packed lamellar like body forms to loosely arranged inactive forms (105, 106, 107).

Our *in vitro* results are supporting the concept that repeated relatively high surfactant doses are needed for treatment of secondary surfactant deficiency in MAS (108). Protein content and composition of surfactant preparations may be critical issues in the design of an artificial surfactant for treatment of ARDS (53, 54). Although KL$_4$ and rSP-C were less sensitive to surfactant inhibition than all other commercially available surfactant preparations, natural surfactant containing all SPs including SP-A and SP-D was even more resistant to loss of biophysical activity by exposure to increasing meconium concentrations. SP-A contributes to increased resistance of surfactant to inactivators (109, 110). Similar effects resulting in increased resistance of surfactant to meconium inactivation have been found after addition of polymeric ionic and non-ionic polymers (111, 112, 113, 107).

4.2. SURFACTANT INACTIVATION BY SILICONE OIL (PAPER II AND V)

4.2.1. *In vitro* effects on surfactant

While assessing a porcine modified natural surfactant at a low phospholipid concentration of 2.5 mg/mL, we were puzzled to note that identical preparations showed excellent biophysical activity—i.e., rapid adsorption with γ_{min} close to zero after a few seconds—when the measurements were done in Göttingen, whereas identical samples had poor surface activity when examined with the same type of equipment in Stockholm. A closer look at our technique revealed that the only difference between the centres was the way technicians filled sample chambers with surfactant. In Göttingen polyethylene pipettes were used, whereas in Stockholm

chambers were filled by means of one-mL syringes, containing rubber, and injection needles (Figure 8); to remove bubbles from the samples, the piston of the syringe was moved up and down five to ten times. The use of syringes resulted in a delayed drop of γ_{min} <10 mN/m depending on the number of aspirations (Figure 9).

Figure 8: Devices for filling the sample chamber of the PBS: Pipette with gel loading tip, two-part syringe and three-part syringe with rubber plunger.
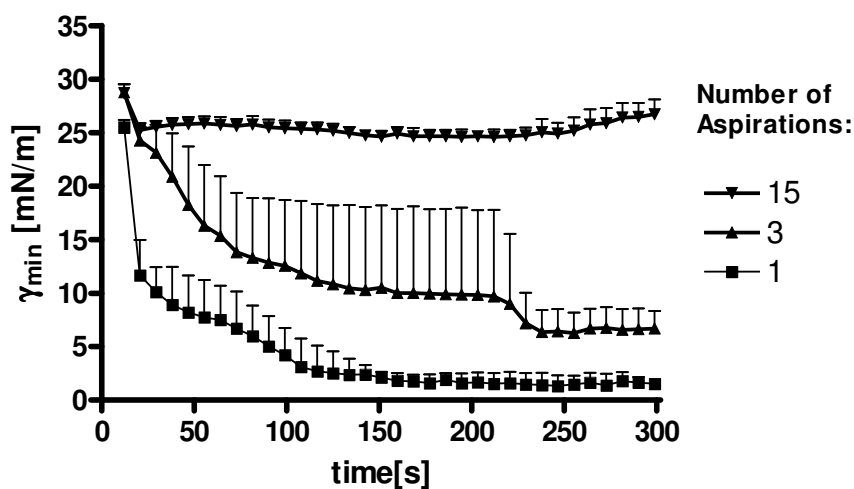

Figure 9: γ_{min} during 300 s of pulsation in the pulsating bubble surfactometer: Curosurf, 80 mg/mL, that was aspirated 3 or 15 times into an Omnifix F three-part syringe and that was subsequently diluted to 2.5 mg/mL. As a control Curosurf, 2.5 mg/mL, which was first diluted and then once aspirated, is shown. Results are expressed as mean ±SD of n=5-6 repeated measurements. $p<0.05$ for 3 vs. 1 aspiration; $p<0.01$ for 15 vs. 1 aspiration (One-way ANOVA followed by Dunnett's post test).

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Further investigations revealed that the syringes were lubricated with silicone oil (SO) in order to run the rubber-coated piston smoothly in the body of the syringe (information provided by B.Braun, Melsungen, Germany). Although the composition and quantity of the oil used are accepted by ISO 7886-1, the international standard for sterile hypodermic syringes for single use, we noted that the silicone lubricant is a potent inhibitor of surfactant function. Similar inhibitory effects were noted in rubber-containing syringes, manufactured by other companies (data not shown). Concentrations as low as 0.1% (v/v) inactivated surfactant.

In fact, by comparing mass spectra of the SO with organic solvent and surfactant, both rinsed in syringes, we showed that silicones are released by rinsing the syringes with surfactant (Figure 10). Mass spectra of silicone showed monomers with m/z intervals of 74, which are typical for polydimethylsiloxanes (PDMS) (114).
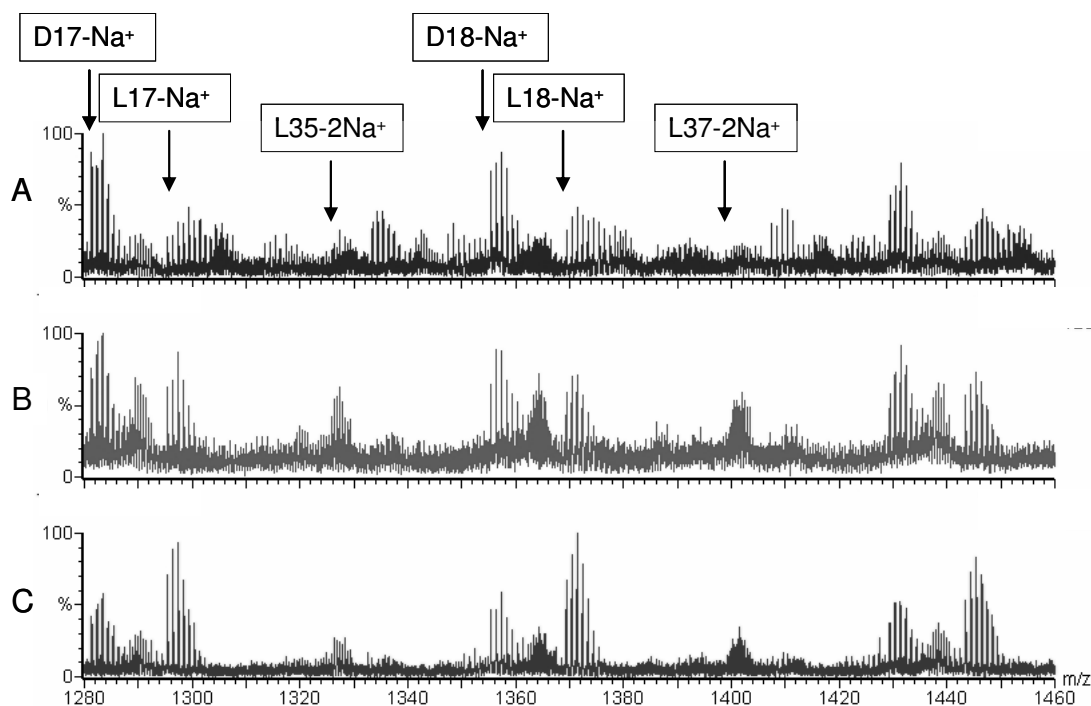

Figure 10: Representative mass spectra of samples after organic extraction of a syringe rinsed with n-heptane (A) compared to a syringe rinsed with Curosurf 2 mg/mL (B) and silicone oil (C) at m/z interval 1280-1460. As an example repeated isotope patterns with the distance of 74 mass units associated to linear (Ln) and cyclic (Dn) polydimethylsiloxanes (PDMS) adducted to Na$^+$ is shown.

The biophysical effects of surfactant rinsed in syringes and of surfactant mixed with SO derived from the manufacturer showed identical characteristics: At low SO/Curosurf

ratios, we found delayed reduction of γ_{min} (Table 3A) and, at high ratios, an inactivation, defined as an increase of γ_{min} after 5 min.

Table 3: Surface tension [mN/m] after adsorption of 2 min (γ_{ads}), minimum (γ_{min}) and maximum (γ_{max}) surface tension after 1 and 5 min of pulsation in a pulsating bubble surfactometer of SO/Curosurf (2.5 mg/mL) (A, B) and SO/Curosurf (10 mg/mL) (C) and a synthetic phospholipid mixture (dipalmitoylphosphatidylcholine/palmitoyl-oleoylphosphatidylglycerol) 10 mg/mL (D) with SO 0, 0.13 and 1.3 mg SO/mg phospholipid. Values are expressed as mean ±SD of 5 to 8 repeated measurements. *: p<0.05 vs. 0; **: p<0.01 vs. 0 (One-way ANOVA followed by Dunnett's post test).

	A			**B**		
	SO/Curosurf 2.5 mg/mL [mg/mg]			SO/Curosurf 2.5 mg/mL [mg/mg]		
	0	0.01	0.04	0	0.13	1.3
γ_{ads}	27.6±1.9	27.0±1.2	27.3±1.2	27.2±0.8	26.1±0.7*	23.6±0.9**
γ_{min} 1'	2,2±0.8	17.5±11**	23.5±1.1**	2.2±0.9	24.1±0.6**	21.6±0.8**
γ_{min} 5'	2.2±0.8	8.0±11	9.7±11	2.7±0.5	12.6±11**	21.1±0.6**
γ_{max} 1'	29.3±2.0	27.8±0.9	27.4±1.0	31.4±1.2	35.9±5.0*	23.5±0.8**
γ_{max} 5'	28.8±2.3	28.8±2.9	29.0±2.2	31.6±1.6	41.7±10**	23.5±0.6*
	C			**D**		
	SO/Curosurf 10 mg/mL [mg/mg]			SO/phospholipids 10 mg/mL [mg/mg]		
	0	0.13	1.3	0	0.13	1.3
γ_{ads}	24.5±0.6	24.0±0.6	21.0±1.3**	74.8±2.0	48.3±8.9**	28.9±2.5**
γ_{min} 1'	1.8±0.8	10.2±9.7*	19.9±1.2**	69.1±2.8	20.2±0.6**	20.6±0.9**
γ_{min} 5'	1.7±0.7	6.3±9.4	19.2±0.8**	58.0±19	20.4±0.6**	20.3±0.7**
γ_{max} 1'	30.3±1.1	25.4±1.0**	22.3±1.1**	73.8±0.6	46.8±5.1**	33.5±1.2**
γ_{max} 5'	30.6±1.9	27.1±2.2**	22.1±0.7**	74.8±1.2	44.8±2.2**	32.0±0.9**

Further biophysical studies on silicone contaminated surfactant showed that an increase of the surfactant concentration prevented an increase of γ_{min}, but still resulted in a decrease of γ_{max}, indicating a mechanism which is, at least in part, reversible (Table 3C). Moreover, addition of silicone oil resulted in a dose dependent decrease of γ_{ads} and γ_{max} (Table 3). Synthetic biophysically poor active PLs, showed a silicone-dose dependent decrease of γ_{ads}, γ_{min} and γ_{max}, indicating infiltration of the PLs film (Table 3D). Similar alterations have been found in surfactant inactivation by other lipid substances as free fatty acids or lyso-phosphatidylcholine (115; 49). In contrast to this

irreversible infiltration, surfactant inactivation by proteins is considered to be reversible by increasing the surfactant concentration (116).

Western blots of the extracted hydrophobic (chloroform/methanol) phase of surfactant resulted in a reduction of SP-B-bands depending on the dose of added SO (Figure 11). Neither signals of SP-C in extracted and non-extracted Curosurf, nor signals of SP-B in non-extracted Curosurf (data not shown) were reduced by addition of SO. Moreover, anti-SP-B blots with extracted and desalted Curosurf suggested reduced migration of the material during electrophoresis, whereas the controls showed SP-B signals at the expected site (~18 kDa). This observation could be explained by binding of SP-B to SO or by denaturation of SP-B at the interface between the two phases during the extraction procedure.

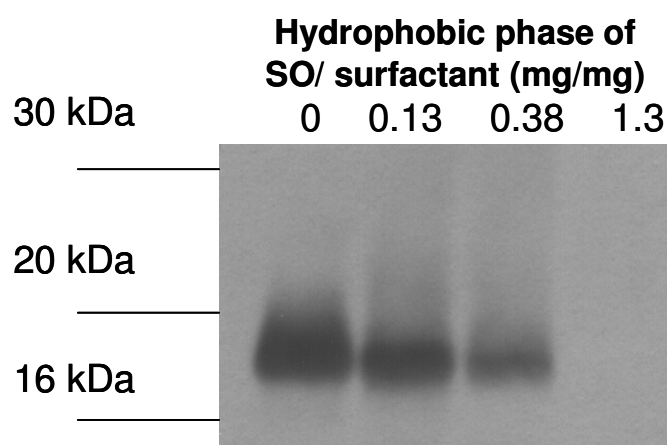|	Chloroform/ methanol phase	Methanol/ water phase	no extraction
SP-C bands	no effect	no signal	no effects
SP-B bands	reduction ≥0.38	no signal	no effects

Figure 11: Anti SP-B *Western* blot of chloroform/methanol phase obtained after extraction of silicone oil (SO)/ surfactant mixtures 0-1.3 mg/mg phospholipid.

4.2.2. Effects of SO contaminated surfacatant *in vivo*

Though *in vitro* results showed impaired biophysical activity after contamination of Curosurf with SO, the compliance of treated animals did not show any significant

impairment. Compared to animals treated with Curosurf only, those receiving SO/Curosurf had an abnormal gas expansion pattern accompanied by changes in inspiration dynamics. T_I of animals was prolonged by SO in a dose-dependent fashion. More remarkably, a SO dose dependent increase of multiple atelectatic lung areas was found, similar to patchy lung expansion as seen in the MAS. This resulted in a dose dependent decrease of *LGV* (Figure 12).
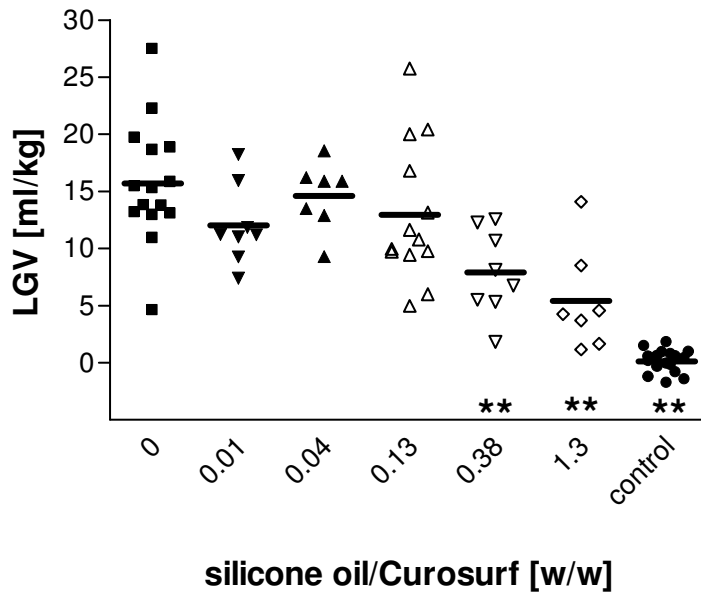

Figure 12: End-expiratory lung gas volumes (*LGV*) and its mean (bar) in animals treated with SO/Curosurf mixed at ratios of 0-1.3 (mg/mg) compared to non-treated controls. **: $p < 0.01$ vs. 0 (One-way ANOVA with Dunnett's post test).

Addition of SO to Curosurf did not increase epithelial damage of the conducting airways, but surfactant treated animals had reduced damage compared to controls as previously demonstrated (71). We speculate that SO infiltrates the surface film in a dose-dependent and non-homogenous manner thus leading to a local loss of ability to reach near zero surface tension during film compression, followed by patchy lung expansion pattern. Reduction of SP-B induced by intratracheal exposure of monoclonal SP-B antibodies caused irregular alveolar collapse and focal desquamation in ventilated near term rabbits (117). Furthermore, compound transgenic mice, in which expression of SP-B was conditionally downregulated, showed focal SP-B deficiency and focal air space enlargement (118). Thus our finding of multiple atelectatic lung fields could be caused by both, surfactant displacement by SO and focal SP-B reduction.

The clinical significance of surfactant contamination depends on a variety of factors as demonstrated in Table 4. At ratios that were maximally achieved in a clinical setting, we did not find the significant effects on *in vitro* surface tension and SP-B-reduction as well as the *in vivo* findings of prolonged T_I and decreased *LGV*.

Table 4: Expected maximum silicone oil/surfactant phospholipid (SO/PL) ratios, when a surfactant at 80mg/mL is aspirated into 1ml, 2ml or 5ml syringes that are either coated with SO according to DIN EN ISO 7886-1 ($\leq$0.25 SO mg/cm²) or B.Braun (0.05-0.07 mgSO/cm²) standard from which 0.1-0.2 mgSO/cm² is dissolved into liquids like surfactant as found by Capes (91).

Syringe (mL)	e.g. surfactant (mL(mg))	Inner surface (cm²)	DIN standard SO (mg)	*Ratio SO/PL*	Braun SO (mg)	*Ratio SO/PL*	Capes: SO (mg)	*Ratio SO/PL*
5	3 (240)	~19	$\leq$4.7	*$\leq$0.02*	1.3	*0.005*	4	*0.016*
2	1.5 (120)	~10	$\leq$2.5	*$\leq$0.02*	0.7	*0.006*	2	*0.017*
1	1 (80)	~8	$\leq$2	*$\leq$0.025*	0.6	*0.008*	2	*0.017*

4.3.   STUDIES ON MIXTURES OF SURFACTANT AND POLYMYXIN B (PAPER III AND VI)

Pulmonary surfactant is inactivated in MAS and neonatal pneumonia. Development of an exogenous surfactant less sensitive to inactivation might be useful treating these diseases. We investigated *in vitro* whether addition of the cationic cyclic membrane cross-linking peptide PxB and/or calcium chloride ($CaCl_2$) to modified porcine surfactant increases resistance to meconium-induced inactivation of surface activity while antimicrobial activity of PxB is maintained. For early prevention of Gram-negative bacterial translocation via the conducting airways and for prevention of bacterial inactivation of surfactant, a combined tracheal instillation of surfactant plus antibiotics could be of use. In further studies, lung function and bacterial proliferation were investigated in near term rabbits treated with PxB/ Curosurf 0, 0.25, 0.5 and 1 % (2.5 mL/kg body weight, corresponding to 200 mg/kg Curosurf). The administered dose of PxB was between 0.5 and 2 mg/kg and corresponds to the recommended daily doses of 2.5 mg/kg/d aerosolised PxB (119).

4.3.1. Surface activity *in vitro* using meconium

These studies further characterised surfactant inactivation by meconium. In addition to the effects described in paper I we found that surfactant inactivation by meconium is reflected by delayed effects on adsorption in the PBS and spreading in the Wilhelmy balance. Presence of $CaCl_2$ and/or $CaCl_2$ plus PxB reduced or counter-balanced effects of meconium on surfactant. Addition of 1% PxB to Curosurf/$CaCl_2$ shifted the dose response curves, measured in the PBS, to the right, *i.e.* a higher meconium concentration was needed for surfactant inactivation (Figure 13). Thus, addition of PxB to Curosurf/$CaCl_2$ further increases resistance to meconium-induced inactivation.
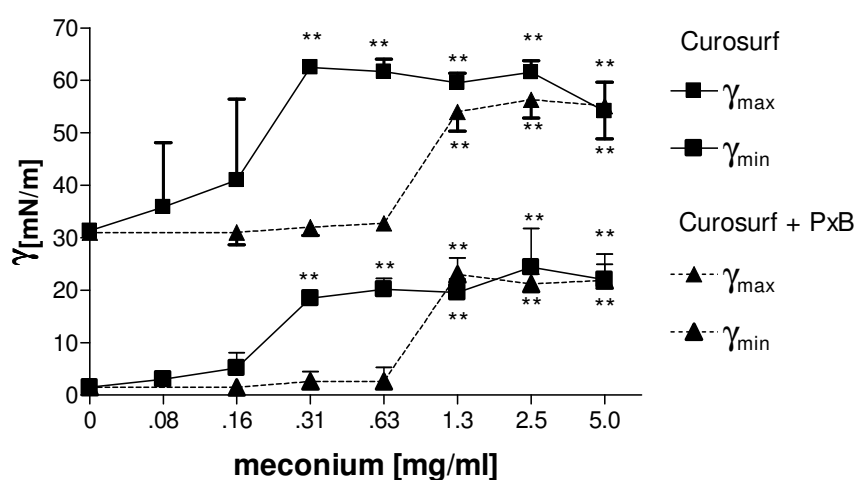
Figure 13: Effects of increasing meconium concentrations on γ_{min} and γ_{max} of Curosurf 2.5 mg/mL and Curosurf 2.5 mg/mL plus 1% (wt/wt) PxB both in presence of 1.5 mM $CaCl_2$. Values were recorded after 5 min of pulsation in the PBS and are expressed as mean +SD of γ_{min} and γ_{max}. **$p < 0.01$ vs. 0 mg/mL meconium (One-way ANOVA with Dunnett's post test); $n = 5$ experiments.

$CaCl_2$ improves film stability of mixtures of extracted lung surfactant and cholesterol (120). However, by comparing surfactant preparations in presence or absence of SP-B and SP-C, the latter compounds seem to be more important for achieving low surface tension and respreading of the monolayers during film compression/decompression than calcium (121).

PxB may connect lipid vesicles thus enlarging the surface-associated surfactant reservoir (122) in the hypophase and making phospholipids readily available for surface adsorption in the pulsating bubble. Moreover, PxB promoted bidirectional transfer of monoionic phospholipids like phosphatidylglycerol, but not of zwitterionic

molecules such as DPPC (123). Enrichment of DPPC, the main component of surfactant, at the air liquid interface of the lung is believed to be a significant function of surfactant proteins (124). We speculate that PxB might contribute to this refinement by sorting the phospholipids.

4.3.2. Bacterial growth *in vitro*

In the present study, meconium alone as well as meconium/Curosurf promoted growth of *E.coli* and GBS (p <0.05), but not of *S.aureus*. Addition of Curosurf/PxB or PxB alone significantly reduced the growth of *E.coli* (p <0.01; Figure 14).
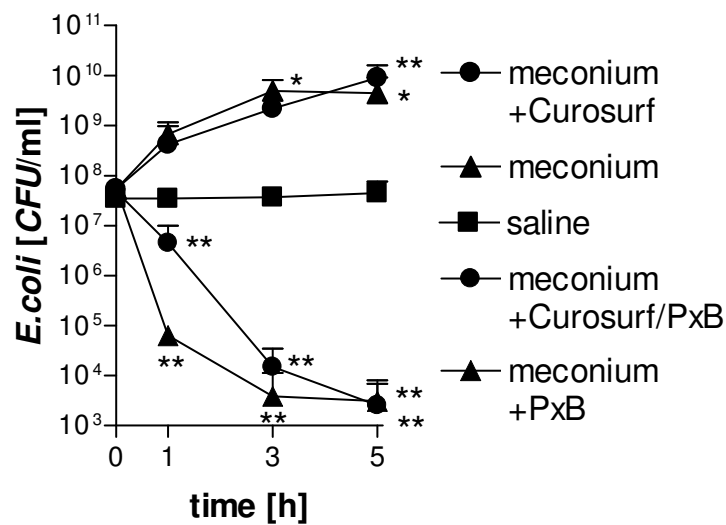
Figure 14: Growth of *E.coli* in saline, meconium, meconium plus Curosurf, meconium plus PxB, and meconium plus Curosurf and PxB. Values are expressed as mean +SD of five experiments. *p < 0.05; **p < 0.01 vs. time = 0 h (One-way ANOVA with Dunnett's post test).

In the absence of meconium, Curosurf at concentrations 1-20 mg/mL showed a bactericidal dose-dependent effect on GBS, but not on *E.coli* or *S.aureus* (125). In rats, intratracheal instillation of *E.coli* combined with meconium increased mortality caused by bronchopneumonia, compared with controls treated with *E.coli* and saline (126). Meconium at concentrations ≥3 mg/mL in amniotic fluid promoted growth of *E.coli* and *Listeria monocytogenes* but reduced growth of *S.aureus* (127). Data from a recent study suggested that meconium counteracts the bacteriostatic effects of amniotic fluid and enhances the growth of GBS more than that of *E.coli*. Furthermore, the proliferation of both bacteria increased with meconium concentration and incubation time (128). Meconium diluted in saline to 20 mg/mL but not saline amplified growth of

E.coli, GBS, *S.aureus*, and several other Gram-positive and Gram-negative bacterial pathogens (129).

4.3.3. Lung function in neonatal *E.coli* pneumonia in rabbits

In general, the compliance of animals with neonatal pneumonia was poor, as previously described. Significant differences were not found. Remarkably, a wide range of compliance was found in all treatment groups. In ARDS with capillary damage due to bacterial or viral pneumonia and other aetiologies reduced compliance and increased work of breathing is found (130). In rats with lung injury due to intratracheal endotoxin instillation an inconsistent response to identical doses was found (39). In ARDS also alterations of the endogenous surfactant pool are found (131). In addition, pulmonary changes caused by the inflammatory reaction may interfere with surfactant production and function. In a clinical study decreased lung compliance in children with ARDS due to pneumonia was associated with decreased levels of SP-A compared to controls of postoperative surgical children (132). Furthermore, endotoxin and Gram-negative bacteria induce similar changes in surfactant function (133). PxB like SP-C binds in a stoichiometric manner to the lipid A moiety of endotoxin (134, 135). This could contribute to our observed poor compliance with a decrease over time for *E.coli* pneumonia controls and surfactant/PxB treated animals. A similar decrease of lung compliance was obtained in endotoxin-induced lung injury in guinea pigs (136). In our study, the dose of administered surfactant per kg body weight is low compared to studies in adults with ARDS caused by sepsis or pneumonia, in which a dose of 300 mg/kg body weight of Alveofact was reported to improve gas exchange (52). In contrast, minimal effects on compliance were found in newborn lambs that were prematurely infected with endotoxin (137).

4.3.4. Bacterial growth *in vivo*

Early onset neonatal sepsis with Gram-negative ampicillin-resistant *E.coli* is an emerging problem among very low birth weight infants. PxB experiences attention in connection with emergence of multi-drug resistant Gram-negative bacteria (138, 139). The main result of the present study is that intratracheal treatment reduces the bacterial count in lungs of animals receiving PxB only or Curosurf/PxB ≥0.5% compared to control animals and those receiving only Curosurf (e.g., Figure 15). A significantly reduced translocation of *E.coli* to the left kidney is found in animals receiving Curosurf/PxB (n=0), compared to control animals and to Curosurf treated animals (p<0.01). Thus, administration of Curosurf/PxB mixtures exerts potent antibacterial

effects and may prevent bacterial translocation from the lung to the systemic circulation.
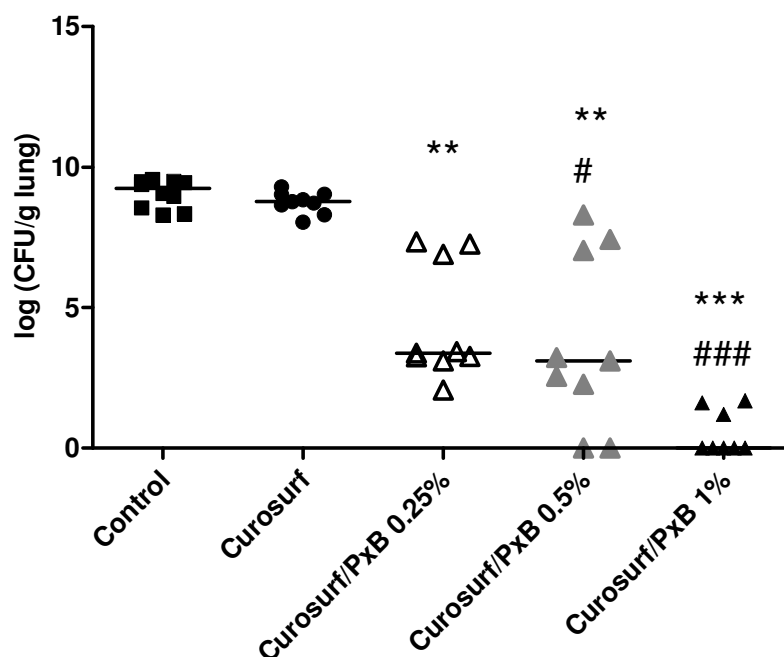

Figure 15: Logarithm of colony forming units (CFU/g lung) in animals receiving saline (control) and Curosurf±PxB (0.25-1%) followed by bacterial inoculation of 10^8/mL *E.coli* (5ml/kg) and ventilated for 240 min with standardised tidal volume of 6-7 mL/kg. Results were corrected by addition of 1 CFU to enable calculation of the logarithm for those showing total bacterial eradication. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ vs. control; #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$ vs. Curosurf (Non-parametric Kruskal-Wallis test with Dunn's post test).

Liposomal incorporation of antibiotics has been used e.g. to increase local antimicrobial effects using tobramycin, which is currently under preclinical investigation (140). In rats with experimental pulmonary *Pseudomonas aeruginosa* infection, treatment with PxB incorporated in liposomes, composed of DPPC and cholesterol, resulted in lower bacterial counts per lung compared with treatment with free PxB or liposomes without PxB (141, 142). The efficacy of PxB incorporation depended on liposome composition (141). The amounts of PxB entering surfactant lipid vesicles after addition to the surfactant suspension, as used in this study, are not known. The PxB concentration used in our study is 100-400fold higher than the minimum *in vitro* inhibitory concentration against *E.coli* of liposomal PxB, encapsulated in DPPC:cholesterol (142).

4.3.5. Histology

Histological analysis demonstrates an inflammatory response with alveolar influx of inflammatory cells in all animals after tracheal instillation of *E.coli* (see e.g. Figure 16).
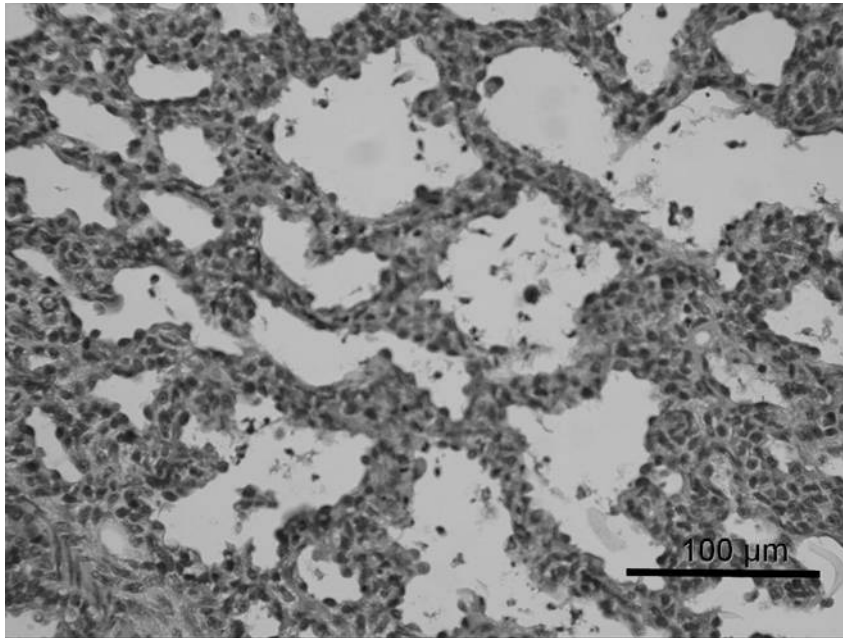

Figure 16: Representative histological lung section of an animal treated with PxB/Curosurf 0.5% and *E.coli*, showing neutrophil influx, alveolar debris and oedema of the alveoli.

Pulmonary inflammation is present, also in PxB treated animals. PxB causes cytolysis of Gram-negative bacteria by inducing pore formation in the membranes. Thus lipopolysaccharide (LPS; endotoxin) of bacterial walls, which is a potent activator of the inflammatory cascade, is still present in the alveoli and as a powerful proinflammatory agent it can lead to increase of vascular permeability, neutrophilic alveolitis and septic shock including ARDS (143, 144). The intensity of systemic inflammatory and physiologic responses to intrapulmonary Gram-negative infection depends on the inoculum size and whether the bacteria are cleared from or proliferate in the lungs (145). The inflammation also might be triggered to a significant degree by mechanical ventilation (146). To reduce ventilator injury in the present study, we decided to use a low standardised *VT* of 6-7ml/kg body weight which is recommended in ARDS (147), but we didn't apply any PEEP. Van Kaam concluded from animal experiments that an open lung ventilation strategy in a model of GBS pneumonia is more important in attenuating inflammatory response than surfactant treatment (148). Lachmann and co-workers showed a reduced bacterial translocation in an ARDS model of GBS pneumonia in animals receiving surfactant and ventilated with an open lung

concept (149). Interestingly, a decreased level of severe inflammation is found in animals receiving Curosurf plus PxB compared to control animals (p<0.05 vs. control). This is in line with the observation that PxB or synthetic derivates (due to high affinity to LPS) have been used in animal experiments and clinical trials to neutralise endotoxin (150, 151). Inflammatory effects may also be reduced by surfactant treatment that suppresses cytokine secretion, mitogen driven proliferation, neutrophil influx and immunoglobulin production (152, 153). We speculate that by optimising the ventilation strategy in our model, the level of inflammation and bacterial translocation might be decreased and early death might be prevented.

4.4. THE IMPACT OF SURFACTANT PROTEIN B AND C ON ALVEOLAR STABILITY AND LONG TIME SURFACE ACTIVITY (PAPER IV)

The effects of different surfactant preparations were investigated *in vitro* in a CBS and *in vivo* using the immature newborn rabbit model. First, SP-B or SP-C was suspended in a PLs mixture to mimic the composition of PLs in natural surfactant regarding disaturated species and PLs classes. Second, native SP-C was replaced with SP-C33 in order to evaluate the effects of SP-B in a completely synthetic mixture. Third, the first phospholipid mixture was replaced with a simple mixture of DPPC/POPG (68:31, w/w). Curosurf treated animals served as controls.
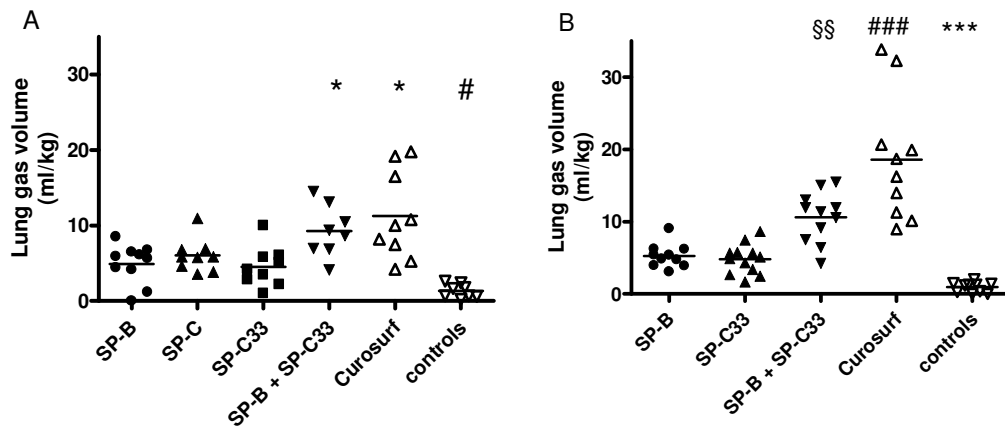

Figure 17: Lung gas volumes in preterm newborn rabbits treated with peptides in either (A) DPPC/PLPC/POPG/PLPE (55:25:15:5, by weight) (80 mg/mL, 2.5 mL/kg), or in (B) DPPC/POPG (68:31, w/w) (80 mg/mL, 2.5 mL/kg), Curosurf (80 mg/mL, 2.5 mL/kg) or in non-treated controls. Levels of statistical significance: *: p <0.05-0.001 vs. SP-C, SP-C33 and SP-B, respectively; #: p <0.05-0.001 vs. all surfactants; ***: p <0.001 vs. SP-B+SP-C33 and Curosurf; ###: p <0.001 vs. all synthetic surfactants; §§: p<0.01 vs. SP-C33 and SP-B (One-way ANOVA followed by Newman-Keuls multiple comparison test); the lines indicate median values.

We found that addition of SP-B to surfactant containing only the synthetic analogue SP-C33 prevents decrease of adsorption property and loss of biophysical activity, as described by the rate of compression needed to obtain a surface tension of 5 mN/m at the 5th cycle *in vitro*. Moreover, addition of SP-B to surfactant containing only SP-C or the synthetic analogue SP-C33 increases *LGV*s *in vivo* (Figure 17). These effects occur in the absence of PEEP and without any effect on *VT*s. Although these effects of native SP-C and synthetic SP-C33 are suboptimal, they are equal. Thus, this suboptimal performance may be due to the lack of SP-B. The increased *LGV*s are not associated with the increased total peptide content of the preparations, as we did not find any significant differences between preparations containing 2% and 4% SP-C33.

Numerous *in vitro* studies using surface balances, PBS and CBS, have shown that SP-B or SP-C mixed with phospholipids show similar activity (see e.g. 154). This has led to a common assumption that they perform similar and partially overlapping functions in alveolar surfactant. In contrast, studies using mice where the genes for SP-B or SP-C have been individually knocked out have indicated a more complex picture. SP-B knockout mice, like humans with inherited deficiency of SP-B, develop fatal respiratory distress shortly after birth (155). The interpretation of these studies is in part complicated by the fact that SP-B knockout mice, like SP-B deficient humans, lack mature SP-C and instead accumulate a late processing intermediate of proSP-C that has very low surface activity in mixture with PLs (156). In addition, SP-B deficiency results in severe alteration of the type II cell, including absence of normal lamellar bodies.

From the results showing increased *LGV*s and the histological findings we conclude that both SP-B and SP-C/SP-C33 are necessary for establishment of alveolar stability in ventilation without PEEP. In addition to the superior effects in premature newborn rabbits, the *in vitro* properties, as measured in the CBS, are better for surfactant containing both SP-B and SP-C33. This applies to adsorption as well as γ_{min} and γ_{max}, and surface area reduction required achieving low surface tension during cycling. Moreover, the surfactant containing both proteins appears to be more stable, as it stays active for up to seven days, while the single protein mixtures lose surface activity already after 1-3 days.

Taken together, this implies that SP-B and SP-C fulfil different functions in alveolar surfactant. The current findings indicate that exogenously delivered surfactant preparations require both SP-B and SP-C, or analogues thereof. This is in line with the

behaviour of SP-B deficient mice and humans, but it is not anticipated from the behaviour of SP-C deficient mice. SP-C knockout mice apparently have normal respiration at birth but develop signs of emphysema and interstitial pneumonitis over time, which suggest that SP-B alone is able to establish low alveolar surface tension and stable terminal airspaces at birth (31). The reasons for the discrepancies between the present findings and those from SP-C deficient mice are not clear. It is possible that isolated porcine SP-B administered via the airways along with a few synthetic PLs species is less efficient than endogenous SP-B secreted into the alveoli together with the native surfactant lipid mixture. SP-B is a complex molecule with several α-helices folded together by intra- and interchain disulphides (157), which makes it conceivable that partial loss of structure can take place during its isolation from surfactant phospholipids.

In the last two decades, several attempts were made to formulate a protein-containing, synthetic surfactant preparation for treatment of respiratory distress. This has not yet resulted in a commercially available product. The synthetic surfactants studied so far have been based on synthetic or recombinant analogues of either SP-B or SP-C (158). All these preparations show promising effects in animal models of lung disease provided that PEEP is applied. However, modified natural surfactants show optimal activity also without PEEP, suggesting that they are functionally superior to synthetic surfactants based on single protein constituents. The present investigation shows that the presence of both SP-C or an SP-C analogue, and SP-B in a binary mixture of synthetic phospholipids is sufficient to establish high *LGV*s in immature newborn rabbits also in the absence of PEEP.

5. CONCLUSIONS

This investigation is one of the first comparing commercially available surfactants in their resistance to inactivation by meconium *in vitro*. New synthetic surfactants containing analogues of SP-B and SP-C are more resistant to inactivation than modified natural preparations. Meconium inactivation can be counterbalanced by increasing the concentration of those surfactant preparations that contain surfactant proteins. Also calcium and the cyclic antimicrobial cross-linking peptide PxB improve resistance of modified porcine surfactant to inactivation by meconium *in vitro*. In mixtures with the modified porcine surfactant and meconium antimicrobial activity of PxB against Gram-negative bacteria is maintained. The prophylactic intratracheal administration of surfactant/PxB mixtures in a model of neonatal *E.coli* pneumonia prevents bacterial growth and bacterial translocation to the systemic circulation. Thus, PxB might be a useful adjunct in treatment of neonatal pneumonia and MAS. Further animal studies are needed to investigate efficacy, safety and toxicity of these mixtures.

By chance, we discovered that silicone oil originating from syringes inactivates pulmonary surfactant *in vitro* and *in vivo*. The oil, which is based upon PDMS, is used to lubricate the syringes and is released by rinsing with surfactant. *In vitro*, the inactivation of surfactant by silicone oil is only in part reversible by increasing the surfactant concentration. *Western* blot studies revealed interference of silicone oil with SP-B. After intratracheal treatment of surfactant mixed with silicone oil, immature neonatal rabbits develop non uniform lung expansion, show retarded inflation during mechanical ventilation and reduced *LGV*s. The clinical use of silicone oil containing syringes in handling exogenous surfactant should be avoided as silicone free syringes are readily available.

In synthetic surfactants the presence of SP-B plus SP-C improves surface activity *in vitro* also during long time surface cycling and is necessary to establish high *LGV*s in ventilated immature rabbits in the absence of PEEP. Determination of *LGV*s is an important additional parameter to identify end expiratory alveolar stability in animal experiments.

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