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An Alternative Model for Efficacy Testing of Exogenous Surfactant using the Isolated Perfused Rat Lung (IPL)

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ABE	Actual Base Excess
AECC	American-European Consensus Conference
ALEC	Artificial lung expanding compound
ALI	Acute lung injury
ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
bw	Body weight
CDS	Container-disperser-spacer
C_{dyn}	Dynamic lung compliance
C_L	Lung compliance
CPA	Continuous powder aerosolizer
CPAP	Continuous positive airway pressure
C_{stat}	Static lung compliance
ddH_2O	Double-distilled water
DI	Deep inspiration
DPPC	Dipalmitoylphosphatidylcholine
EEP	End-expiratory pressure
EIP	End-inspiratory pressure
EthD-1	Ethidium homodimer-1
FiO_2	Fraction of inhaled oxygen
GSD	Geometric standard deviation
HALI	Hyperoxic acute lung injury
HCO_3^-	Hydrogen carbonate
HE	Hematoxylin and eosin
HMD	Hyaline membrane disease
I:E	Inspiration to Expiration ratio

INT	2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride
IPL	Isolated perfused rat lung
ITEM	Institute for Toxicology and Experimental Medicine
LDH	Lactat dehydrogenase
LISA	Less invasive surfactant administration
MIST	Minimal invasive surfactant therapy
MMAD	Mass median aerodynamic diameter
mRNA	messenger ribonucleic acid
n.d.	Not detectable
NNMU	*N*-Nitroso-*N*-methylurethane
P	Pressure
P_a	Extravascular pressure
PAP	Pulmonary artery pressure
PEEP	Positive end-expiratory pressure
P_i	Intravascular pressure
PIP	Positive end-inspiratory pressure
pO_2	Partial pressure of oxygen
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
P_t	Transmural vascular pressure
RDS	Respiratory distress syndrome
R_L	Airway resistance
RLL	Rat lung lavage model
rSP	Recombinant surfactant protein
R_V	Vascular resistance
sO_2	Saturation of blood with oxygen
SP	Surfactant Protein
t	Time
V	Volume
VP	Venous pressure
V_T	Tidal volume
WADT	Wet aerosol delivery tube

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1. Introduction

Pulmonary surfactant is an amphipathic structure that is produced by alveolar type II cells. Biophysically, surfactant enables a reduction of the surface tension in the alveolar epithelium, making it essential for respiration as it enables normal gas exchange through the alveolar-capillary barrier. Inhibition of pulmonary surfactant function or surfactant deficiency can have very serious health consequences. The most prominent diseases associated with a dysfunction of pulmonary surfactant are the respiratory distress syndrome (RDS) and the acute respiratory distress syndrome (ARDS). In RDS, the pulmonary surfactant production in premature infants is insufficient because of immature lung development (MA and MA, 2012). In fully developed lungs, an inhibition of endogenous surfactant function can lead to ARDS. This is commonly caused by sepsis, multiple blood transfusions, or major fractures or other (PEPE et al., 1982). Both syndromes lead to severe consequences such as alveolar collapse or pulmonary edema formation (ROY et al., 2012; SCHILLER et al., 2001). In both syndromes, the maintenance of oxygen saturation is the primary target of therapies.

Besides adequate mechanical ventilation of the patients, much effort was put into the development of surfactant replacement therapies. The administration of exogenous surfactant has repeatedly been shown to lead to a reduction in surface tension (BANASCHEWSKI et al., 2015; KOLOMAZNIK et al., 2015; SALITO et al., 2015). Today, around a dozen of exogenous surfactants have been tested successfully as effectively improving oxygen saturation and reducing morbidity and mortality in preterm infants with RDS. Although significant improvements of oxygenation or reduced mortality in ARDS patients could not be shown until now, research into the extension of the indication area of exogenous surfactant preparations for the treatment of ARDS continues (DAVIDSON et al., 2006; RAGHAVENDRAN et al., 2011; ZHANG et al., 2013). Exogenous surfactant drugs must have high surface activity to show high clinical relevance. This can be achieved on the basis of a variety of sources: Besides synthetic surfactant preparations that do not contain any animal-

derived proteins or lipids, also organic solvent extracts from processed lung tissue are used for several exogenous surfactant preparations (RAGHAVENDRAN et al., 2011; WILLSON and NOTTER, 2011). Moreover, extracts of lavaged endogenous lung surfactant from different animal species are applied in surfactant replacement therapies.

The biophysical activity of exogenous surfactant preparations such as their surface tension lowering abilities and surface adsorption qualities can be determined using *in vitro* methods. The most commonly used methods are the Langmuir–Wilhelmy balance, the pulsating bubble surfactometer, the captive bubble surfactometer, or the constrained sessile drop (ZUO et al., 2008). In order to determine the physiological effects of exogenous surfactant preparations on lung mechanics or improved gas exchange, animal models are used. Prior to patient administration, the efficacy of every exogenous surfactant batch is tested *in vivo* in models of impaired lung function (ZUO et al., 2008). Test animals with impaired lung function are in critical condition due to complications resulting from unstable blood pH regulation or increased permeability of the alveolar-capillary membrane which may cause premature decease. Against the background of animal welfare, the aim of this thesis is the establishment of a more gentle procedure for efficacy testing of exogenous surfactant preparations. To this end, test animals are exsanguinated under deep anesthesia before the lung-heart bundle is removed. The lung is then ventilated and perfused *ex vivo* while the extracted organ receives pulmonary function impairing treatment. Exogenous surfactant is then administered and changes in the respiratory function are assessed.

2. Literature

2.1. Anatomic Aspects of the Mammalian Lung

Mammalian lungs are connected by the trachea and are embedded flexibly in the pleural cavities. Towards the thoracic cavity, the lungs are covered by a smooth lining, the tunica serosa. Inside the thoracic cavity, this lining is also known as pleura. The pressure between those two linings is sub-atmospheric, which is of utmost importance for respiration.

The mammalian lungs are subdivided into several lung lobes. The right lung of humans consists of the upper, the middle and the lower lobe. These lobes are further divided by lung segments, ten of which make up the right lung. The left lung is divided into the upper and the lower lobe. Additionally, the upper lobe has a projection, the lingula. The lingula can be seen as an anatomic parallel to the middle lobe of the right lung (ZILLES and TILLMANN, 2010).

There are noteworthy anatomical differences between the human and the rat lung. In contrast to the human lung, the right lung of rats consists of four lobes: lobus cranialis pulmonis dexter, lobus medius pulmonis dexter, lobus accessories pulmonis dexter, and lobus caudalis pulmonis dexter (KOMÁREK, 2000). The left lung is only made up of a single lobe, the pulmo sinister which can be subdivided into the pars cranialis and the pars caudalis (KOMÁREK, 2000). This is important to keep in mind when evaluating the deposition of substances in lung areas. The left lung in mammalians is generally smaller compared to the right lung as the heart projects farther to the left than to the right lung (GREY, 2000).

The lung of mammalians is part of the respiratory system. Within this system, air is transferred within the conducting zone from the pharynx to the trachea and then into the left or right bronchus. These air ducts branch out from two primary bronchial tubes at the carina of the trachea. Further down in the conducting zone, bronchial

tubes branch out into secondary and tertiary bronchioles until they branch out into respiratory bronchioles and end in the respiratory zone. Respiratory bronchioles transfer air into alveoli where gas exchange takes place.

Warm-blooded animals require high rates of oxygen uptake for a sufficient oxygenation of the circulatory system. This necessitates an immense lung surface. The mammalian lung is an organ which accounts for 6% of the whole body volume. Its main characteristic is a large inner surface that is continuously in contact with the environment (CREUWELS et al., 1997). The dimension of the large surface is realized via membranous sacs, which are divided into alveoli. The alveolar parenchyma consists of small sacs that in turn increase the surface of the lung enormously (CREUWELS et al., 1997). Thus, 1 cm^3 of lung tissue is made up of an alveolar surface of 300 cm^2. In human, the alveolar surface area during expiration amounts to 80 m^2, while it expands to 120 m^2 during inspiration (ZILLES and TILLMANN, 2010).

The function of the alveolar surface is the exchange of gas between the ambient air and the blood (CREUWELS et al., 1997). Alveoli have an epithelium and are covered by capillaries. Oxygen diffuses from the outwardly oriented alveolar epithelium into the capillaries (CREUWELS et al., 1997). In the opposite direction, carbon dioxide leaves the capillaries and diffuses into the alveoli (CREUWELS et al., 1997). From here, carbon dioxide leaves the respiratory tract and is transported through the conducting zone to the upper airways.

The alveolar epithelium contains three types of pneumocytes. Besides the existence of macrophages in the alveolar epithelium, the majority of pneumocytes are type I alveolar cells. These take part in the process of gas exchange at the air-blood barrier. Type I cells have only very few organelles and consist of multiple cytoplasmatic plates that represent the surface area for gas exchange in the alveoli (WARD and NICHOLAS, 1984). Alveolar type II cells can divide and act as progenitors for alveolar type I and type II cells (WARD and NICHOLAS, 1984). Additionally, type II cells secrete pulmonary surfactant and release it continuously by exocytosis into the alveolus (CREUWELS et al., 1997). Pulmonary surfactant is a

fluid that enables the lowering of surface tension inside the alveoli. Surface tension on the alveolar surface originates from the attraction between the molecules in the humid environment of alveoli (CREUWELS et al., 1997). Without pulmonary surfactant, alveoli would collapse during expiration which would lead to partial or even complete lung collapse.

2.2. Respiratory Mechanics

Normal spontaneous respiration is based on a combination of active muscle contractions of the diaphragm during inspiration and a passive muscle relaxation phase during the expiratory phase of breathing (GRINNAN and TRUWIT, 2005). By muscle contraction, tension is built up at the diaphragm and sub-atmospheric interpleural pressure is created. This negative pressure is transferred to the lungs, causing negative pressure in the alveoli (BENJAMIN, 2016). The resulting pressure gradient is sufficient to allow air to flow into the airways (GRINNAN and TRUWIT, 2005). The inflow of air stops when the pressure in the alveoli reaches supra-atmospheric pressure, followed by the expiratory phase of respiration (DRAZEN, 1984). An elastically recoil is caused by a decrease in muscle tension, which results in the creation of positive alveolar pressure (BENJAMIN, 2016). This causes carbon dioxide-rich oxygen mixture to be exhaled from the respiratory tract. In contrast to the reversing alveolar pressure, pleural pressure remains sub-atmospheric throughout the entire respiration process (MEAD et al., 1970). The expiratory phase ends when the inwardly recoil of the lung has compensated outward tension of the chest wall. At this time, the alveolar pressure is adjusted to atmospheric pressure and a respiratory cycle is completed (DRAZEN, 1984).

The filling of the lungs with air thus depends on two different pressure differences. On the one hand, the pressure difference between alveolar and atmospheric pressure is required to overcome the airway resistance R_L and to transport air through the trachea (UHLIG and TAYLOR, 1998). On the other hand, the difference

between the pleural and the atmospheric pressure reflects the combination of the pressure (P) needed to overcome R_L and the pressure needed to inflate the bronchi and small airways with a specific volume (V) of air (DRAZEN, 1984) This force is described by elasticity (E). This relationship is depicted by Uhlig and Taylor by the equation below, in which I stands for the inertia of the gas molecules and is largely negligible (UHLIG and TAYLOR, 1998):

$$\mathrm{P} = EV + R\frac{dV}{dt} + I\frac{d^2V}{dt^2}$$

Equation 1

2.2.1. Resistance

In addition, the reciprocal value of E is referred to as lung compliance (C_L) which describes the elasticity of the lung parenchyma (UHLIG, 1992). Hence, Equation 2 can be applied:

$$\mathrm{P} = \frac{1}{C_L}V + R_L\frac{\mathrm{dV}}{\mathrm{dt}}$$

Equation 2

R_L is a parameter that correlates with the upper airways of the respiratory tract, whereas C_L is rather associated with the elasticity of the widely branched parenchyma. R_L and C_L can be determined by simultaneous measurement of transpulmonary pressure, volume, and flow (dV/dt). Using a pneumotachometer, the air flow rate dV/dt and can be measured while P is analyzed by a pressure sensor P. In this context, the maximum calculated volume presents the tidal volume (V_T). During inspiration and expiration, an isovolumetric point can be determined. This term can be used for determination of R_L (UHLIG and Taylor, 1998), according to Equation 3:

$$R_L = \frac{\Delta \mathrm{P}}{\Delta \mathrm{dV/dt}}$$

Equation 3

The airway sizes, the airflow direction (inspiration or expiration), as well as the flow regime have influence on the expression of R_L (DRAZEN, 1984). Initially, the alveolar parenchyma has a large cross-sectional area. As air flows towards the mouth during expiration, said cross-sectional area decreases more and more. Hence, changes in the trachea and the first generations of the upper airways account for over 90% of pulmonary resistance to airflow under normal circumstances (DRAZEN, 1984). Therefore R_L is relatively insensitive to changes in smaller airways and the alveolar parenchyma. An increase in pulmonary resistance can occur for instance by accumulation of fluids in the upper airways (DRAZEN, 1984). Besides an increased production of mucus as a consequence of e.g. cigarette smoking or air pollution, the impact of bronchial edema formation can also be seen in a R_L increase (DRAZEN, 1984). In contrast, R_L decreases with increasing inflation pressure, which is also why pulmonary resistance in adults is three to five times lower compared to that found in children (BRISCOE und DUBOIS.A.B, 1958). High inspiratory pressure increases the volume of inhaled air, as the airways are extended (DRAZEN, 1984). This results in distention and lengthening of bronchi (BRISCOE and DUBOIS.A.B, 1958). While lengthening has an increasing effect on lung resistance, the increase in diameter of bronchi is more prominent (DRAZEN, 1984).

2.2.2. Compliance

In contrast to the calculation of R_L at isovolumetric points, C_L is determined at points of full inspiration or complete expiration, when dV/dt is zero (UHLIG and TAYLOR, 1998):

$$C_L = \frac{\Delta V}{\Delta P}$$

Equation 4

Through the creation of pressure-volume diagrams, C_L can be represented graphically (GRINNAN and TRUWIT, 2005). In pressure-volume diagrams, C_L is the relation between two pressure-volume differences at two different plot points. In this way, the expression of C_L differs, depending on the flow direction and ranges of V and P over which C_L is computed. As can be seen in pressure-volume curves of full inflation, the relation between V and P is almost linear during the mid-volume range in deflation (DRAZEN, 1984). In this range, C_L is less influenced by changes in V and P and is therefore called static compliance (C_{stat}) or quasi-static compliance (GRINNAN and TRUWIT, 2005).

Volume and pleural pressure can be measured throughout the complete respiratory cycle. By this, the ratio of volume changes to pressure differences between full inspiration and expiration is determined. This is the dynamic compliance (C_{dyn}) (DRAZEN, 1984).

C_{dyn} reflects the elasticity of the lower airways and at the same time includes the force which is necessary for overcoming the air flow resistance R_L. Airway closure, which mostly occurs in small airways, reduces the amount of alveolar tissue that is able to perform gas exchange (DRAZEN, 1984). Hence, C_{dyn} is especially reduced in the small airways during bronchoconstriction (UHLIG and Taylor, 1998). In addition, especially C_{stat} is affected by edema formation or fibrosis.

2.3. Pulmonary Surfactant

In 1929 Kurt van Neergard considered the functioning of the lung tissue in a completely new way. Until then, the retraction force of the lungs was explained solely by the elasticity of lung fibers. He was the first to state that the force of surface

tension must have much greater influence on the retraction force of the alveolar epithelium than tissue elasticity (VAN NEERGARD, 1929).

The Young–Laplace equation, which was defined in the early 1800s, gave an explanation for the phenomenon of surface tension and its effects on pressure decreases at a boundary surface separating two phases of matter (WILLSON and NOTTER, 2011). When the law of Young and Laplace is applied to alveoli, the generated decrease in pressure that is needed to inflate alveoli is directly proportional to surface tension and inversely proportional to the alveolar radius (WILLSON and NOTTER, 2011).

Especially the radius of curvature of the alveoli has a distinct influence on the expression of surface tension between the alveolar epithelium and the alveolar air (VAN NEERGARD, 1929). Van Neergard suggested the existence of surface-active substances that might reduce pulmonary surface tension which would be physiologically insurmountable in normal breathing.

In the 1950s Richard Pattle discovered foam in the trachea of rabbits with lung edema (PATTLE, 1955). He also examined healthy lungs by cutting and squeezing them underwater or performing a saline lavage, revealing similar occurrences of foam. Pattle suggested that the stability of such foam was due to an insoluble surface layer on the bubbles. Furthermore, he found out that the layer could be enzymatically digested.

In 1959, Mary Ellen Avery et al. first described the association of infant respiratory distress syndrome (RDS), formerly known as hyaline membrane disease (HMD), and surfactant deficiency (AVERY and MEAD, 1959). Gribetz and Avery found that excised lungs showing hyaline membranes contain less gas and show impaired static compliance compared to lungs of normal newborns when inflated with positive pressure of up to 35 cmH_2O (GRIBETZ et al., 1959). Thus, she concluded that the hyaline membrane disease “is associated with the absence or delayed appearance of some substance which in the normal subject renders the internal surface capable of

attaining a low surface tension when lung volume is decreased” (AVERY and MEAD, 1959).

It was Avery’s clinical supervisor Clements Smith who then measured the surface tension-decreasing characteristics of several lipid fractions. In their studies, phospholipids had the biggest impact on surface tension. They showed that the activity of synthetic dipalmitoylphosphatidylcholine (DPPC) was similar to that of phospholipids isolated from bovine lungs (KLAUS et al., 1961). Using fetal rabbit lungs, the production of DPPC during lung development could be shown as well as DPPC secretion into the alveolar space (GLUCK et al., 1967).

In 1973, King et al. isolated proteins from canine surface active material (KING et al., 1973). Besides albumin and IgG, the presence of a non-serum protein with a strong affinity for phospholipids and a very large percentage of hydrophobic amino acid residues was shown in their study. This demonstration of the existence of protein content in surfactant was essential for the recognition that proteins might be important constituents of surfactant (CREUWELS et al., 1997).

Nowadays, pulmonary surfactant is generally known for its stabilizing effect in respiration whilst preventing alveolar collapse (GRIESE, 1999). Lung surfactant lowers the surface tension in the alveolar epithelium and improves the uniformity of inflation in all alveoli (CHRISTMANN et al., 2009). The maximal adsorption of lung surfactant is achieved in its tubular myelin structure (NOTTER, 2000). Moreover, surfactant can prevent antigen uptake by particle-binding and thus enhances cellular immune responses (WINKLER and HOHLFELD, 2013).

2.3.1. Composition

Surfactant from the extracellular compartment can easily be collected by bronchoalveolar saline lavages. When comparing surfactant of different mammalian species, the compositions of surfactant shows striking similarities (GOERKE, 1998).

Surfactant mainly consists of lipids (90 - 95%), with proteins representing only a small part (5-10%) (HAAGSMAN and VAN GOLDE, 1991). About 90 - 95% of total lipid consists of phospholipids and 5 to 10% is made up of neutral lipids such as cholesterol and cholesterol esters plus diglycerides/triglycerides (CHRISTMANN et al., 2009; NOTTER, 2000). 75% of phospholipids consist of phosphatidylcholine, while its main part is made up of 50 - 55% DPPC and other saturated phosphatidylcholines and about 45% consist of unsaturated phospholipids (CHRISTMANN et al., 2009; NOTTER, 2000). Around 20% of the surfactant phospholipids comprise anionic phospholipids such as phosphatidylglycerol (12%), phosphatidylethanolamine (5%), and phosphatidylserine (2%). Besides, surfactant contains other phospholipids such as phosphatidylinositol (4%), and sphingomyelin (1.5%) (CHRISTMANN et al., 2009; NOTTER, 2000; WILLSON and NOTTER, 2011).

The surface tension lowering activity of surfactant is not only determined by DPPC and other phospholipids. The biophysical function of pulmonary surfactant is also defined by proteins. The surfactant proteins (SP) SP-A, SP-B, and SP-C influence the surface activity of lung surfactant, while the surfactant protein SP-D does not affect surfactant activity (NOTTER, 2000). Two of these apoproteins, SP-A and SP-D, are large and water-soluble, while SP-B and SP-C are small and very hydrophobic (JOHANSSON et al., 1994).

SP-A is the most abundant apoprotein in pulmonary surfactant (BOGGARAM et al., 1988). The hydrophilic apoprotein can bind two to three Ca^{2+}. This might neutralize the environment of the apoprotein near neutral pH. SP-A binds phospholipid vesicles and contributes to the exchange of phospholipids (CAJAL et al., 1998). The apoprotein increases the aggregation and promotes surface activity of the phospholipids (EFRATI et al., 1987). In this context, SP-A promotes the formation of ultrastructurally tubular myelin-like surfactant structures (NOTTER, 2000).

SP-D is very different from the other surfactant apoproteins in that SP-D does not influence the biophysical function of pulmonary surfactant. SP-D is a collagenous glycoprotein that is hydrophilic and has structural analogy to SP-A (NOTTER, 2000). Thus, SP-D is found neither in tubular myelin meshworks of surfactant nor in lamellar

bodies (CROUCH et al., 1992). Like SP-A, SP-D is a member of the collectin family of host defense proteins (MASON et al., 1998). It is, like SP-A, also involved in a range of immune functions including viral neutralization, clearance of bacteria, fungi and apoptotic and necrotic cells, down regulation of allergic reactions and resolution of inflammations (KISHORE et al., 2006).

SP-B and SP-C are hydrophobic apoproteins that are processed post-translationally from larger precursor forms that are not present in alveoli, into small, active peptides (JOHANSSON et al., 1994). Both hydrophobic apoproteins show strong interactions with phospholipids (NOTTER, 2000).

SP-B mainly exists as a homodimer and is strongly positively charged. It can selectively disrupt and remove anionic and unsaturated lipid species from the alveolar surface film (JOHANSSON et al., 1994). SP-B increases phospholipid aggregation, and helps the insertion of phospholipids into surface films (OOSTERLAKEN-DIJKSTERHUIS et al., 1991a). SP-B can also form higher oligomers through intermolecular cysteine-linked sulfhydryl bridges (TAKAHASHI et al., 1990). SP-B can interact with phospholipid headgroups via its polar residues (NOTTER, 2000). The surface activity of endogenous surfactant is best when both SP-B and SP-A are present, as a combination of SP-A and SP-B with calcium is required for the conversion of secreted endogenous surfactant to tubular myelin-structures in the alveolar lining (JOHANSSON et al., 1994).

The monomeric form of SP-C is much smaller than SP-B and features extreme hydrophobicity (GLASSER et al., 2001). It can also form oligomers or α-helix structures and accelerate the adsorption of lipid bilayers to an interfacial monolayer (PASTRANA et al., 1991). Due to its high hydrophobicity, SP-C is thought to locate in the interior of the phospholipid bilayer in its α-helix structure (NOTTER, 2000). Besides the interaction of SP-C with phospholipids through strong hydrophobic forces, SP-C’s positively charged amino acid residues can interact with phospholipid headgroups (NOTTER, 2000). SP-C promotes fusion of phospholipid vesicles and thus helps the insertion of phospholipids into interfacial films (CREUWELS et al., 1993; OOSTERLAKEN-DIJKSTERHUIS et al., 1991a).

2.3.2. Metabolism

Surfactant is synthetized in the endoplasmatic reticulum of alveolar type II cells in the lung parenchyma and can be assigned to two different pools (CHRISTMANN et al., 2009; CREUWELS et al., 1997). The intracellular surfactant compartment is found inside alveolar type II cells. Within these, surfactant is stored in lamellar bodies until it gets released into the alveolar space (HAAGSMAN and VAN GOLDE, 1991). After the release from lamellar bodies, surfactant is secreted into the alveolar space. In this extracellular compartment, surfactant is transformed into tubular myelin which forms monolayers that line the alveolar epithelium (CREUWELS et al., 1997).

In detail, pulmonary surfactant is produced inside alveolar type II cells (ASKIN and KUHN, 1971). Within the pneumocytes, the main surfactant phospholipid DPPC is produced inside endoplasmatic reticula from its precursor glucose and then transported with the Golgi system to lamellar bodies (ASKIN and KUHN, 1971; JOHANSSON et al., 1994; VAN GOLDE et al., 1988). Inside lamellar bodies, surfactant is stored as phospholipid bilayers that are organized in concentric twists known as intra-alveolar tubular myelin (ASKIN and KUHN, 1971; JOHANSSON et al., 1994). The surfactant proteins SP-A, SP-B, and SP-C are also stored inside these lamellar bodies, though the presence of SP-D could not be shown (OOSTERLAKEN-DIJKSTERHUIS et al., 1991b; VOORHOUT et al., 1992; WALKER et al., 1986). Existence of surfactant proteins, including SP-D, could furthermore be demonstrated inside club cells (VOORHOUT et al., 1992; WALKER et al., 1986).

Surfactant is secreted into the alveolar hypophase by exocytosis (RYAN et al., 1975). Exocytosis of surfactant can be influenced by different factors. Reinforced breathing or ventilation increases surfactant secretion by a cholinergically mediated mechanism (OYARZUN and CLEMENTS, 1977). Also, beta-adrenergic mediators and prostaglandins are involved in increased surfactant release during extended lung ventilation (FISHER et al., 1985; OYARZUN and CLEMENTS, 1978). Moreover, protein kinase C and cyclic AMP-dependent protein kinase, leukotrienes, and Ca^{2+} influence exocytosis of surfactant from alveolar type II cells (DOBBS et al., 1986;

ROONEY and GOBRAN, 1988; SANO et al., 1985). The process of surfactant secretion is negatively regulated under feedback control (JOHANSSON et al., 1994). In this context, SP-A itself has been shown to inhibit DPPC secretion (DOBBS et al., 1987). Moreover, actin filaments are very likely to be involved in the movement and exocytosis of lamellar bodies (TSILIBARY and WILLIAMS, 1983).

It has been reported that every hour up to 40% of surfactant in lamellar bodies is exocytosed into the alveolar hypophase (NOTTER, 2000). However, the synthesis rate of pulmonary surfactant is less than the secretion rate from alveolar type II cells which has been assumed to be indicative of surfactant recycling. Thus, reuptake by type II pneumocytes is a major factor for the elimination of surfactant from the alveolar hypophase (ENNEMA et al., 1984; POST and VAN GOLDE, 1988). Only 10-15% of surfactant is degraded by macrophages and an even smaller subset of about 2-5% is eliminated via exhalation (CHRISTMANN et al., 2009). After uptake into type II cells, surfactant components are transported to the lamellar bodies and are then reconnected to newly synthesized surfactant before being released again (NOTTER, 2000).

In the alveolar hypophase, surfactant can occur in several formations. Surfactant aggregates can be separated into large aggregates that consist of tubular myelin a light subtype, consisting of small unilamellar vesicles (GOERKE, 1998). The ability to lower surface tension of surfactant aggregates is a function of their size, with large aggregates showing higher surface activity (GUNTHER et al., 1999). Moreover, the content of phospholipids and proteins varies between aggregates. As large surfactant aggregates contain more apoprotein, it is assumed that they are very active and are made up of recently released surfactant components (GUNTHER et al., 1999; NOTTER, 2000). In contrast, smaller surfactant aggregates show less surface tension lowering abilities and might consist of surfactant that has undergone multiple cycles (GUNTHER et al., 1999).

Endogenous surfactant can form cross-hatched networks of phospholipid bilayers called tubular myelin (WILLIAMS, 1977). These networks consist of phospholipids that are organized by the surfactant proteins SP-A and SP-B under the influence of

Ca^{2+} ions (SUZUKI et al., 1989). Endogenous surfactant in the formation of tubular myelin is associated with high adsorption at air-liquid interfaces (NOTTER et al., 1986). During each inspiration, tubular myelin and other surfactant aggregates have to spread to the monolayer at the air-liquid interface in less than 1 s for rapid adsorption of the monolayer (JOHANSSON et al., 1994).

2.4. Impairment of Pulmonary Surfactant Function

The necessity of lung surfactant implies that an inactivation or inhibition of pulmonary surfactant will lead to severe consequences. In contrast to other biological inactivation processes, the primary negative impact of alveolar surfactant impairment does not result in cellular responses. Rather, lung surfactant inactivation or inhibition processes have a biophysical result that is a decrease in surfactant's surface activity at the alveolar epithelium (NOTTER, 2000).

The function of alveolar surfactant can be impaired for example by endogenous inhibitors like plasma and blood proteins that biophysically reduce the surfactant film activities (HOLM et al., 1999)The best-known inhibitors of lung surfactant are plasma proteins like fibrinogen, fibrin monomers, hemoglobin or albumin (HOLM and NOTTER, 1987; NOTTER, 2000).

Ingestion of exogenous substances such as oleic acid or cholesterol can lead to an impairment of surface tension lowering effects (HALL et al., 1992a). Similarly, fluid fatty acids as well as lysophospholipids penetrate the interfacial film and fluidize it (HOLM et al., 1999). This leads to an increase in surface tension. Moreover, endothelial permeability can be increased by acids (NOTTER, 2000). As regards neonates, ingestion of meconium is another very important factor in the impairment of surfactant function. Meconium contains several pulmonary surfactant inhibitors such as tissue proteins or fatty acids that can cause severe breathing problems in newborns (CLARK et al., 1987; MOSES et al., 1991).

Alveolar surfactant can also be chemically impaired by degradation. This can be caused by proteases such as neutrophil elastase, phospholipases, and reactive oxidants during pulmonary inflammation processes (HOLM et al., 1991; PISON et al., 1989). In the latter case, amino acids in surfactant proteins as well as unsaturated fatty acids in surfactant lipids can be oxidized (GILLART et al., 1998). Also several antibodies that bind to surfactant proteins can alter the surface active function enormously (KOBAYASHI et al., 1991; ROBERTSON et al., 1991).

Large surfactant aggregates are mostly selectively depleted by metabolic alterations in lung injury. All of these alterations lead to decreased surface activity of endogenous surfactant. Alterations to alveolar type II cells may furthermore result in reduced surfactant synthesis and thus lead to surfactant deficiency (NOTTER, 2000).

The effects of pulmonary surfactant impairment can result in increased alveolar surface tension and reduced gas exchange at the air-blood interface. The magnitude of surfactant function inhibition depends on both surfactant and inhibitor composition and concentration (NOTTER, 2000). As a consequence, all of the above mentioned factors leading to reduced surface activity of lung surfactant can give rise to impaired breathing. In the contemporary literature, it is generally established that an impairment of surfactant function is associated with RDS and acute respiratory distress syndrome (ARDS) (HALLIDAY, 2008; MACINTYRE, 2000; WILLSON and NOTTER, 2011; WISWELL, 2001).

2.5. Pulmonary Surfactant Dysfunction

Lung surfactant dysfunction is a very rare lethal genetic condition with unknown etiology in which at least one functional component of surfactant is depleted or the composition of surfactant is altered (ILKOVICH et al., 2014). All of these abnormalities lead to impaired surfactant functionality and result in neonatal alveolar proteinosis and respiratory failure (HAMVAS et al., 1995; NOTTER, 2000). Lung surfactant dysfunction is a disorder which in most cases leads to a deficiency in SP-B

production (CHETCUTI and BALL, 1995). The SP-B encoding gene SFTPB, as well as mutations in the genes for surfactant proteins-C (SFTPC), the ATP binding cassette member A3 (ABCA3), or the thyroid transcription factor (NKX2.1) are involved in disorders of the surfactant metabolism (HAMVAS, 2010).

Surfactant deficiency is refractory to exogenous surfactant administration and assisted ventilation (MCFETRIDGE et al., 2009). The only cure for infants suffering from surfactant deficiency is a lung transplantation which results in reconstitution of pulmonary surfactant function (HAMVAS et al., 1995).

2.6. Respiratory Distress Syndrome (RDS)

RDS occurs in premature infants within the first few minutes of life when infants exhibit symptoms related to severe respiratory failure (VERMA et al., 2006). These symptoms can include nasal flaring, retraction, cyanosis, tachypnea, or expiratory grunting (NOTTER, 2000; RODRIGUEZ, 2003; SHELLEDY and PETERS, 2016).

RDS was previously known as HMD. HMD refers to hyaline-rich membranes that were found in pathological examinations of alveoli of RDS-affected premature infants. In 1947 it was observed that the extremely high surface tension might be the leading cause for difficulties in aeration of alveoli in HMD (GRUENWALD, 1947). Several years later, surfactant deficiency was linked to HMD (PATTLE, 1955). After Avery and Mead found out that bronchoalveolar lavage fluids from infants suffering from HMD had lower surface tension lowering effects, it was shown that HMD is linked to a deficiency in a surface active substance (AVERY and MEAD, 1959). Today, it is known that the primary cause for RDS is a diminished capacity of alveolar type II cells in pulmonary surfactant production (NOTTER, 2000).

Even though many improvements in the treatment and understanding of RDS have been made during the last decades, RDS is still the most common respiratory disorder and cause of death in neonates (LIU et al., 2010; RODRIGUEZ, 2003). Low

birth weight is the main reason for perinatal mortality (MCCORMICK, 1985). Even if most premature babies survive at present, there is an increased risk of undesirable developments in the nervous system and patients have a high risk for respiratory complications such as RDS (GOLDENBERG et al., 2008). Around 83% of all infants with a very low birth weight between 501 and 1000 g born before the 28th gestational week suffered from congenital respiratory insufficiency of prematurity or RDS (HACK et al., 1991; KEEN and PEARSE, 1985). The incidence for RDS decreases with higher birth weight and the gestational age (NOTTER, 2000). In a multi-center cohort study with a total number of 1765 births, 67% of infants with a birth weight between 501 and 1500 g were noted to have RDS and / or respiratory insufficiency of prematurity, depending on the definition used in the centers (HACK et al., 1991). Premature babies that were born between the 32nd and 34th gestational week had an incidence for RDS of 10 - 20%, while RDS only occurred in under 5% of infants born after the 35th week of gestation (HACK et al., 1991). Besides the gestational age and birth weight, the prevalence of RDS is related to other factors. For instance, the incidence of RDS is lower in black preterm infants (HULSEY et al., 1993). Also, maternal diabetes is strongly correlated with RDS (TAEUSCH and BALLARD, 1998). Besides maternal hypertension and drug abuse, selective cesarean section and severe birth asphyxia can promote RDS development (LIU et al., 2010; NOTTER, 2000; RIMAR et al., 2014; RODRIGUEZ, 2003). Moreover, maternal-fetal infection, premature rupture of membranes, maternal thrombophilia and male sex are strongly correlated with RDS in neonates (KALTOFEN et al., 2015; LIU et al., 2010; RIMAR et al., 2014; RODRIGUEZ, 2003).

The number of premature infants, born at less than 37 weeks gestational age has increased significantly in recent decades in developed countries. This is mainly due to the fact that more and more premature births are recorded and more preterm infants are born from multiple artificially fertilized ova (GOLDENBERG et al., 2008). Administration of antenatal glucocorticoids such as dexamethason up to three days before birth increases the number of alveolar type II cells that effectively produce pulmonary surfactant (CROWLEY, 1995). This atenatal therapy seems to be synergistic together with postnatal exogenous surfactant treatment for the lung

development of premature infants with RDS (HACK et al., 1991; NOTTER, 2000; RODRIGUEZ, 2003).

Surfactant deficiency is the leading cause for RDS. As the lung is entirely developed by the end of the second trimester of the pregnancy, alveolar type II cells can only be created after this point (NOTTER, 2000). However, the full number of pneumocytes and sufficient production of lung surfactant is only reached after 32 weeks of gestation. Newborns with RDS experience increased breathing effort and suffer inter alia from chest wall retractions (RODRIGUEZ, 2003). Arterial blood gas analysis can show hypoxemia and hypercapnia that can cause respiratory acidosis and systemic metabolic acidosis (BROUILLETTE and WAXMAN, 1997; RODRIGUEZ, 2003; WARLEY and GAIRDNER, 1962). Pathognomonic radiology findings show alveolar overdistension or alveolar collapse which result in hypoinflation and superimposed air bronchogram (RODRIGUEZ, 2003). Moreover, development of pulmonary edema is associated to RDS (NOTTER, 2000).

In the absence of lung injury, RDS subsides in less than a week, given sufficient pulmonary surfactant production (NOTTER, 2000). In contrast, lung injury impedes the recovery from RDS. Infants suffering from complications associated with RDS need medical treatments. This can include treatment with exogenous surfactant preparations. The course of disease in these infants is very much related to the entirety of the treatment and is not simply a reflection of surfactant replacement therapy alone. Nevertheless, the beneficial effects of surfactant treatment for the prevention or cure of RDS could be shown in several studies (RODRIGUEZ, 2003; SOLL and OZEK, 2009; WILLSON and NOTTER, 2011).

2.7. Acute Respiratory Distress Syndrome (ARDS)

The acute respiratory distress syndrome (ARDS) used to be known as adult respiratory distress syndrome in past decades. Today, the name ARDS refers to the rapid onset of the disease (RANIERI et al., 2012). The acute respiratory distress

syndrome can affect patients in all age groups such as full-term newborns, infants, children, and adults (NOTTER, 2000).

After the original definition of ARDS was introduced by Ashbaugh et al. in 1967, in which no uniform patient groups could be defined, various definitions for ARDS circulated (RANIERI et al., 2012). In 1994, the long accepted definition of ARDS was developed by the American-European Consensus Conference (AECC) (BERNARD et al., 1994). In this definition, ARDS was described with the acute onset of hypoxemia with bilateral infiltrates on frontal chest X-ray with no evidence of pulmonary arterial hypertension (BERNARD et al., 1994). However, the AECC definition had some ambiguities with regard to criteria such as the acute onset of ARDS, or the dimension of oxygen saturation at different positive end-expiratory pressure (PEEP) settings.

The current Berlin definition of the clinical syndrome of ARDS was defined in 2011 (RANIERI et al., 2012). According to this definition, ARDS is a diffuse inflammatory lung injury with an acute onset within one week of the primary insult (RANIERI et al., 2012). ARDS leads to increased pulmonary vascular permeability, an increase in lung weight, and loss of aerated lung tissue (RANIERI et al., 2012). The Berlin definition divides ARDS into three severity levels which are based on the Horowitz quotient (RANIERI et al., 2012). This quotient describes the ratio of arterial oxygen (pO_2) to the fraction of inhaled oxygen (FiO_2). Since the ratio of pO_2 /FiO_2 strongly depends on the PEEP used in ventilation settings, a PEEP ≥ 5 cmH_2O is presupposed in all ARDS severity levels (FERGUSON et al., 2004; RANIERI et al., 2012).

According to this definition, mild ARDS is defined by a Horowitz quotient between 200 mmHg < pO_2 /FiO_2 ≤ 300 mmHg, while moderate ARDS has an oxygenation index after Horowitz between 100 mmHg < pO_2 /FiO_2 ≤ 200 mmHg (RANIERI et al., 2012). Severe forms of ARDS have a Horowitz quotient of pO_2 /FiO_2 ≤ 100 mmHg (RANIERI et al., 2012).

From meta-analyses of clinical trials, five clinical symptoms have emerged that are most likely to trigger ARDS development within 72 h after onset of the primary condition. With descending ARDS-incidence, these are septic syndrome (47%), emergency transfusions (45%), major fractures (44%), pulmonary contusion (38%), and aspiration of gastric contents, meconium or other chemicals (31%) (PEPE et al., 1982). Pepe et al. also found out that near-drowning, pancreatitis, and prolonged hypotension were not related to ARDS onset. A decade later, this order of ARDS-causing conditions was nearly completely confirmed by Hudson et al., the only exception being that near-drowning was found to lead to ARDS more often than pulmonary contusion and aspiration. Additionally, major fractures only led to ARDS development in 23% of the patients (HUDSON et al., 1995). Other causes of ARDS include cardiopulmonary bypass, disseminated intravascular coagulation, immunosuppression, drug overdose or infections of different origins (WARE and MATTHAY, 2000). ARDS can also arise during intensive care and can be hyperoxia- or ventilator-induced (NOTTER, 2000).

Principles of ARDS therapy primarily comprise maintenance of oxygen saturation and gas exchange by adequate support of oxygen transport and mechanical ventilation (NOTTER, 2000; PETTY and ASHBAUGH, 1971; WARE and MATTHAY, 2000). Potential benefits of exogenous surfactant administration in the treatment of ARDS is widely discussed (HAFNER and GERMANN, 1999; LOPEZ-HERCE et al., 1999; RAGHAVENDRAN et al., 2011; ZHANG et al., 2013). During the support phase, further pulmonary injury in the shape of fluid overload, oxygen toxicity or infection must be prevented or treated.

2.8. Exogenous Surfactant

Several substances and pathologic conditions can lead to an inhibition of lung surfactant activity. These inactivation processes can be remedied by increasing surfactant concentrations. This is referred to as inhibition resistance (NOTTER,

2000). With regard to means of therapy for surfactant inactivation, it is possible to take advantage of the phenomenon of inhibition resistance. The concept of exogenous surfactant development is based on this idea.

A few years after the discovery of natural lung surfactant, it was assumed in 1959 that lung surfactant deficiency is responsible for RDS (AVERY und MEAD, 1959). First attempts in treating preterm neonates suffering from RDS started with application of 0.25% microaerosolized DPPC, which is the main component of lung surfactant, in a mixture of propylene glycol and water (ROBILLARD et al., 1964). However, the administration of DPPC by inhalation was not found to be effective in RDS treatment, mainly due to inadequate size distribution of the generated aerosol particles (ROBILLARD et al., 1964). Moreover, the deficiency of DPPC compared to recombinant surfactant concerning the lowering of surface tension had not been researched sufficiently at the time (WILLSON and NOTTER, 2011).

For more than another decade, further development of exogenous surfactant was shelved as pulmonary ischemia came into focus as a cause for RDS. It was believed that ischemia presented a more prominent functional problem in RDS than hyaline membranes or pulmonary atelectasis (CHU et al., 1967). The year 1980 saw the first successful endotracheal administration of exogenous surfactant to ten preterm neonates, eight of whom survived, showing improved gas exchange and resolved radiological abnormalities (FUJIWARA et al., 1980). Nowadays, exogenous surfactant therapy is the gold standard for the treatment of RDS. Since the first exogenous surfactant suspension Exosurf® was approved for intratracheal installation at all gestational stages by the FDA in 1990, mortality rates in premature infants could be reduced significantly (WILLSON and NOTTER, 2011).

2.8.1. Composition of Exogenous Surfactant Preparations

The group of exogenous surfactants includes a range of pharmaceuticals which differ in their composition of lipids and proteins and thus demonstrate varying efficacy in

RDS treatment. In general, exogenous surfactant preparations can be classified in synthetic, animal lung-derived, or natural lung surfactant preparations (WILLSON and NOTTER, 2011).

Group 1) Synthetic surfactant preparations do not contain animal lung-derived material. This group comprises, among others, recombinant protein-based surfactant preparations like Venticute® (iusupultide), *Takeda Pharmaceutical, Ōsaka, Japan*, and protein-free synthetic surfactants like Exosurf® (colfosceril palmitate), *GlaxoSmithKline, London, UK*, the artificial lung expanding compound ALEC® (pumactant), *Britannia Pharmaceutical, Surrey, UK*, or Aerosurf® (formerly known as Surfaxin®) (lucinactant), *Discovery Laboratories, Warrington, PA, USA.*

Group 2) Bovine or porcine lung-derived extracts from minced lungs or saline lung lavages in organic solvents that may contain synthetic additives such as bovine-derived Surfacten® (Surfactant-TA), *Mitsubishi Tanabe Pharma, Osaka, Japan,* Survanta® (beractant), *AbbVie, North Chicago, IL, USA,* or Curosurf® (poractant alfa), *Chiesi USA, Cary, NC, USA*.

Group 3) Natural lung surfactants that derive from bovine lung lavages in organic solvents such as Infasurf® (calfactant), *ONY, Amherst, NY, USA,* bLES® (bovine lung extract surfactant), *BLES Biochemicals, London, Canada*, and Alveofact® (bovactant), *Lyomark Pharma GmbH, Oberhaching, Germany*).

Animal-derived surfactant preparations contain surfactant phospholipids and one or both of the hydrophobic surfactant proteins SP-B and SP-C while the hydrophilic surfactant proteins SP-A and SP-D are removed by organic solvent extraction (WILLSON and NOTTER, 2011). Furthermore, surfactant preparations from lung tissue-derived extracts can contain cellular protein fragments and / or cellular lipids (WILLSON and NOTTER, 2011). Whether or not SP-A is necessary for the surface active function of endogenous surfactant is a matter of debate (NOTTER, 2000). In mice, SP-A gene knock-out influences, i.e. the formation of tubular myelin, but no difference in survival or pulmonary function were found (KORFHAGEN et al., 1996).

However, the presence of the hydrophobic apoproteins SP-B and SP-C together with phospholipids leads to surfactant activity that is nearly equivalent to that of extracted endogenous surfactant (NOTTER, 2000). If either SP-B or SP-C is added to synthetic phospholipids, surface tension lowering abilities are reached that are strongly resemble mixtures in which both apoproteins are present (HAWGOOD et al., 1987; HOLM et al., 1996; NOTTER, 2000; OOSTERLAKEN-DIJKSTERHUIS et al., 1991a). The ability of SP-B and SP-C to facilitate adsorption in exogenous surfactant mixtures correlates with their function in fusion and disruption of phospholipid bilayers (CREUWELS et al., 1993; NOTTER, 2000; OOSTERLAKEN-DIJKSTERHUIS et al., 1991a). Additionally, in surfactant film spreading surfactant proteins B and C exhibit equal abilities (NOTTER, 2000; TANEVA and KEOUGH, 1994; WANG et al., 1996). However, surfactant protein B has a binding capacity for lipid vesicles to interfacial films that is roughly four times higher than that of SP-C (NOTTER, 2000). SP-B exhibits superior activity to surfactant protein C in Wilhelmy balance experiments (WANG et al., 1996). Nevertheless, *in vivo* a surfactant formulation solely containing SP-C as apoprotein was shown to have the same activity as a modified bovine-derived surfactant preparation, Surfacten® (Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) (HAFNER et al., 1998a; HAFNER et al., 1994). Even if bovine derived surfactants contain both surfactant proteins B and C, the content and the ratio of these proteins in each of the surfactant batches varies as the composition depends on the donor cow lung (HAFNER et al., 1998a). Häfner et al. showed that recombinant surfactant protein (rSP)-C was equally as efficient in restoring lung function as bovine-derived surfactant in an animal model of ARDS (HAFNER et al., 1994).

2.8.2. Surfactant Replacement Therapy

Great expectations were placed on the development of synthetic lung surfactant because of clear advantages concerning the production of batches with equal efficacy, no risk of prion transmission, and independence of religious concerns with

bovine- or porcine-derived surfactants (WILLSON and NOTTER, 2011). However, the development of synthetic lung surfactant has proven to be extremely challenging as the effectiveness continually fell short of natural-derived surfactant preparations.

In premature babies with respiratory distress syndrome, the clinical response to treatment with surfactant containing SP-B and SP-C is much faster than in RDS patients treated with protein-free synthetic surfactant (JOHANSSON et al., 1994). A significantly reduced improvement of lung function with protein-free-only surfactant formulations was also seen in clinical trials. Hence, protein-free synthetic surfactants like Exosurf® or ALEC® are no longer used because of considerably reduced efficacy in comparison with existent animal-derived surfactants (RAGHAVENDRAN et al., 2011). In the treating of RDS it was shown that the protein-free exogenous surfactant formulation Exosurf® (colfosceril palmitate) showed lower efficacy in lung function improvement compared to apoprotein-containing Infasurf® (calfactant) (HUDAK et al., 1996; HUDAK et al., 1997; WILLSON and NOTTER, 2011).

Up to now, exogenous surfactant preparations are not able to reduce ARDS-related mortality and also the improving effect on oxygenation in ARDS patients could not clearly been shown in clinical trials (ZHANG et al., 2013). The insufficient effect of exogenous surfactants in ARDS-patients might in part be related to the composition of exogenous surfactant formulations. In children with secondary ARDS, an improvement in oxygen saturation could be found in those suffering primarily from systemic or pulmonary diseases (LOPEZ-HERCE et al., 1999). Contrary to that, after the treatment with the exogenous surfactant preparation Curosurf® (poractant alfa) no improvement in oxygenation was found in children with ARDS resulting from cardiac diseases (LOPEZ-HERCE et al., 1999).

The inactivation processes described in 2.4 for the inhibition of endogenous lung surfactant can also be applied to inactivation of exogenous surfactant formulations. However, some surfactant preparations have been shown to overcome the drop in activity upon increasing the surfactant concentration. In this context, the addition of apoproteins to protein-free surfactant preparations such as Exosurf® showed a slight improvement of resistance to inactivation with raised exogenous surfactant

concentration (HALL et al., 1992b; NOTTER, 2000). This study showed that an addition of SP-B and SP-C enhances the surface activity of DPPC more than additives such as tyloxapol or hexadecanol (HALL et al., 1992b).

Even if the exact mechanisms of recycling of exogenous surfactant remain unclear, it is known that uptake of exogenous surfactant in alveolar type II cells occurs (JOBE, 1988). In this context, it could be shown that Survanta®, as well as the exogenous surfactant preparations Exosurf® and ALEC® were taken up from the alveolar hypophase into type II cells (ASHTON et al., 1992; HALLMAN et al., 1994; IKEGAMI et al., 1989). As for endogenous surfactant, the endurance of surfactant in alveoli is longer for newborns than for adult animals as surfactant uptake in type II cells and recycling rates in newborns are more efficient (IKEGAMI and JOBE, 1998; JACOBS et al., 1982). The incorporation of exogenous surfactant into the recycling process for endogenous surfactant has beneficial physiological long-term effects and improves lung function after administration. Moreover, exogenous surfactants can influence the improvement of lung function by combing with endogenous surfactant in the alveolar hypophase (NOTTER, 2000).

2.8.3. Administration

The standard procedure of RDS prevention and treatment in preterm neonates has been a combination of mechanical ventilation, oxygen treatment, and administration of artificial lung surfactant. Usually, neonates are treated by invasive instillation of surfactant suspensions. For this treatment, endotracheal intubation as well as laryngoscopy are needed, both of which pose substantial risks for neonates and should be avoided whenever possible as immature lungs of preterm infants are very injury-prone (MORLEY et al., 2008; NARCHI and CHEDID, 2015).

Nowadays, antenatal glucocorticoid treatment, noninvasive ventilatory support for spontaneously breathing preterm infants such as nasal continuous positive airway pressure (CPAP) and administration of exogenous surfactant are preferred in order

to minimize the danger of lung injury (JOBE and BANCALARI, 2001; MORLEY et al., 2008; NARCHI and CHEDID, 2015). Using CPAP, less invasive surfactant administration (LISA) or minimal invasive surfactant therapy (MIST) is enabled. In these strategies, surfactant can be delivered intratracheally through a fine catheter without causing lung injury through intubation or mechanical ventilation (KRIBS et al., 2015; LISTA et al., 2015).

As animal studies showed heterogeneous distribution of instilled surfactant which resulted in poor clinical response (VAN DER BLEEK et al., 1993), a new strategy for aerosolization of surfactant was developed. The generation of aequeous surfactant suspensions is limited by the low achievable concentration of the surfactant aerosol and the need for secondary aerosol dilution as the nebulizing system is integrated into the ventilator circuit (POHLMANN et al., 2013). The generation of a highly concentrated surfactant aerosol from dry surfactant powder which can be supplied into the ventilation system very proximally to the patient without dilution by gas flow should help to avoid low surfactant concentration problems (POHLMANN et al., 2013). This study also employed dry powder aerosolization to achieve sufficient surfactant delivery and lung deposition.

Usually, intratracheal instillation of liquid surfactant suspensions is the method of choice for RDS/ARDS treatment. This method is associated with a variety of risks, but nebulized surfactant showed only little success due to inefficient aerosol deposition in the lungs (PILLOW and MINOCCHIERI, 2012). Nebulization of surfactant formulations is limited by the dilution of phospholipids in the surfactant formulation. In contrast to this method, very high surfactant aerosol concentrations can be achieved using dry surfactant powder aerosolization (POHLMANN et al., 2013).

2.9. Animal Models for Exogenous Surfactant Efficacy Testing

Surfactant dysfunction or deficiency can be emulated in various animal models. Research in animal models for the investigation of the physiological impact of exogenous surfactant preparations has been indispensable up until the present (NOTTER, 2000). Thus, data derived from animal models serves as a basis for successful arrangements in clinical trials for exogenous surfactant testing in RDS or ARDS patients.

For the sensible planning of *in vivo* trials to analyze treatment options in RDS and ARDS, recording of the oxygenation status is often a very important parameter. Consequently, the use of large test animals can be reasonable as more blood samples can be obtained from the same individual to analyze the course of gas exchange improvements resulting from the tested treatment.

Also for the understanding of RDS and ARDS pathogenesis, animal models are a link between clinical findings and laboratory research for investigation of physiological mechanisms. Firstly, hypotheses from clinical studies can be investigated in the animal model. Secondly, findings from *in vitro* systems can be validated in animal models in order to check their relevance *in vivo*, before performing experimentation on humans (MATUTE-BELLO et al., 2008).

As exogenous surfactant preparations consist of lipids and, as an optional addition, of proteins, the effectiveness of exogenous surfactant preparations has to be ensured in a batch by batch manner. In principle, two different animal models, namely preterm models and secondary surfactant deficiency animal models in adult test animals can be used for efficacy testing under subnormal lung function conditions.

2.9.1. Preterm models

In preterm models, immature animals are delivered by Caesarean section several days before the natural birth date. Preterm test animals have underdeveloped lungs with very little or no endogenous surfactant production. For this reason, preterm models are also called primary surfactant deficiency models. After delivery, test animals are intubated and hand-ventilated or mechanically ventilated with positive pressure (NOTTER, 2000). Respiratory settings are then adjusted and administration of the exogenous test surfactant is initiated. Depending on the objective of the study, surfactant can be administered as a suspension intratracheally or as an aerosol. Subsequently, improvement of lung function, lung compliance and improvement in oxygenation are assessed. Studies in preterm animals are commonly followed up for several hours or until death, whichever occurs first (IKEGAMI and JOBE, 2002; NOTTER, 2000).

Animal models of prematurity used for studying surfactant activity require a certain gestational age in order to ensure a level of lung maturity in which surface active function is expressed to a certain degree. Even if the majority of the airway expansion occurs postnatal in most mammalians, the human lung matures earlier in gestation than those of other species (HARDING and PINKERTON, 2015; NOTTER, 2000). The alveolation of the human lung tissue is almost completed by the 40th week of gestation, which is 65% of term (NOTTER, 2000). Contrary to that, ovine lungs are almost completely surfactant deficient at this stage of gestation (NOTTER, 2000). Rabbit lungs, for instance, have only produced about 8% of lung surfactant at 96.8% of term (ROONEY et al., 1976).

As regards small animal models, premature rabbits are frequently used (BOHLIN et al., 2005; CALKOVSKA et al., 2016; ENHORNING et al., 1973; SIEW et al., 2011). Therefore, rabbit fetuses are born by Caesarean section at day 27 or 28 of gestation. At this stage of pregnancy, lungs are not completely developed and have only a small amount of alveolar type II cells that can only produce very low levels of pulmonary surfactant (NOTTER, 2000). Studies in premature rabbit lungs are used to

study efficacy of surfactants in reverse surfactant deficiency (ROBERTSON and ENHORNING, 1974). Premature rabbits were used to show that exogenous surfactant can be delivered by intratracheal instillation and can improve pulmonary function, thus extending the lifespan of premature rabbits (ENHORNING et al., 1973; NOTTER, 2000).

The most comprehensive data on the effectiveness of exogenous surfactants is acquired from studies with large animals. Large preterm animal models include e.g. premature rhesus monkeys, baboons, and premature lambs or porcine species (CUTZ et al., 1978; DAVIS et al., 1998; MAETA et al., 1988). Large preterm animal studies provide a direct connection between laboratory research and clinical use of surfactant treatment in infants with RDS. A frequently used large animal model of prematurity is the lamb (DANI et al., 2014; MAZELA et al., 2012; MUSK et al., 2015; VAN ZYL and SMITH, 2013; WOLFSON et al., 2012). For exogenous surfactant testing, lambs are born by Caesarean section between 120 and 135 days of gestation (MAZELA et al., 2012; NOTTER, 2000; VAN ZYL and SMITH, 2013; WOLFSON et al., 2012). Lambs of 120-125 days of gestation are almost completely surfactant deficient and therefore allow for the collection of data less confounded by interactions of administered exogenous surfactant with endogenous surfactant (BRUMLEY et al., 1967; NOTTER, 2000). However, these studies are more demanding as preterm lambs at this gestational stage would not survive without surfactant administration, even if maximal respiratory support is given (NOTTER, 2000). Lambs at gestational ages between 132 and 135 days can survive for least some hours without surfactant administration, as they have already produced endogenous surfactant (BRUMLEY et al., 1967; NOTTER, 2000).

The treatment with exogenous surfactant before delivery has also been analyzed (GALAN et al., 1993; GALAN and KUEHL, 1992; HALLMAN et al., 1997). In these preterm studies, exogenous surfactant was administered intra-amniotically to preterm rabbit fetuses between the 23rd and 27th day of gestation (GALAN and KUEHL, 1992; HALLMAN et al., 1997). It could be shown that small amounts of in utero-administered exogenous surfactant was absorbed into the lungs (6.6%) while 45.9%

were transported to the gastrointestinal tract (HALLMAN et al., 1997). Nevertheless, the administered exogenous surfactant altered the pulmonary function of preterm rabbits, showing lower opening pressures (GALAN and KUEHL, 1992). These experiments were also conducted on preterm baboon fetuses (GALAN et al., 1993). One day before delivery by Caesarean section, exogenous surfactant was administered intra-amniotically at day 136 or 137 of gestation (GALAN et al., 1993). The in utero treatment with surfactant improved the clinical outcome but could not prevent RDS completely (GALAN et al., 1993). However, none of the studies were able to show intra-amniotic surfactant administration to be more effective than treatment after birth (OSTRZENSKI et al., 2006). Due to the additional, potentially heightened risks associated with this invasive method, intra-utero surfactant administration was not taken into consideration for RDS treatment in infants.

2.9.2. Secondary Surfactant Deficiency Models

Adult animal models are also used for studies on surfactant dysfunction or deficiency. In this context, characteristics and mechanisms of ARDS are presented and these animal models should aid the investigation of ARDS therapies. An animal model of ARDS should reproducibly present the pathological changes of the illness as well as improvements over time (MATUTE-BELLO et al., 2008). Currently, no animal model exists that can reproduce all characteristics of ARDS found in patients. Nevertheless, the results obtained from animal models serve to further the understanding of key elements of the inflammatory response to pulmonary diseases in humans (MATUTE-BELLO et al., 2008). One of the biggest problems in modeling ARDS is the diversity of effects in the lung tissues elicited by the various primary insults such as sepsis. The management of ARDS includes different settings of mechanical ventilation and several kinds of medication (MATUTE-BELLO et al., 2008; VLAHAKIS and HUBMAYR, 2005). Thus, the complexity of animal models is increased immensely by the need to incorporate additional variables such as treatment modalities in the experimental designs (MATUTE-BELLO et al., 2008). Several adult animal models

exist for the testing of the effectiveness of exogenous surfactants for the treatment of ARDS. Because of mature lung development in adult test animals, these models are also known as secondary surfactant deficiency models.

ARDS can be induced in adult animals by intravenous infusion of fluid fatty acids such as oleic acid. This leads to a disruption of alveolar and capillary endothelia which results in severe edema formation (ASHBAUGH and UZAWA, 1968; SHAH et al., 1997; ZHU et al., 1998). Oleic acid furthermore leads to surfactant inactivation and inflammation (HALL et al., 1992a; NOTTER, 2000).

Infusion or instillation of antibodies that are directed against the lung tissue or against components in lung surfactant cause ARDS and surfactant dysfunction in test animals (LACHMANN et al., 1987; NOTTER, 2000; SUZUKI et al., 1988).

Bacterial infections or toxins like LPS can also be used to induce ARDS. These models reflect the clinical cause for ARDS, induced by sepsis. Here, toxins are infused intravascularly or instilled intratracheally (HOFFMANN et al., 1991; LUTZ et al., 1998; TASHIRO et al., 2001). Toxins lead to intrapulmonary shunting and decreased pO_2 levels. In addition to surfactant dysfunction, toxins lead to inflammation and edema formation, which is expressed, for instance by decreased compliance (NOTTER, 2000).

Besides bacterial inflammation, several viruses can also cause pulmonary inflammation. Influenzavirus A, respiratory syncytial virus, or Sendai virus can lead to an onset of ARDS with induction of alveolar-capillary membrane damage and surfactant inactivation (NOTTER, 2000; VAN DAAL et al., 1992a; VAN DAAL et al., 1992b; VAN SCHAIK et al., 2000).

Exogenous surfactant therapy is also used in newborns, suffering from meconium aspiration or in patients who were exposed to acid aspiration (AL-MATEEN et al., 1994; COLVERO et al., 2008; ZUCKER et al., 1992). Aspiration in animal models is provoked e.g. by instillation of meconium, hydrochloric acid, or a mixture of betaine hydrochloride and pepsin (AL-MATEEN et al., 1994; COLVERO et al., 2008; LAMM and ALBERT, 1990; STROHMAIER and SCHLAG, 1993). Besides inactivation of

pulmonary surfactant and alteration of the alveolar tissue, aspiration of caustic substances also leads to tracheal damage in patients (NOTTER, 2000).

Another substance that leads to lung injury with several characteristics of ARDS is *N*-Nitroso-*N*-methylurethane (NNMU). Subcutaneous injection of NNMU leads to severe impairment of gas exchange, pulmonary edema, neutrophilic influx into the alveolar space and alterations in endogenous surfactant (CRUZ and MOXLEY, 1998). Lung injuries that originate from NNMU were shown to be alleviated by exogenous surfactant administration (LEWIS et al., 1991).

Formation of pulmonary edema and the treatment of decreased gas exchange with exogenous surfactant formulations can be investigated in an animal model of neurogenically caused edema. In animal models, bilateral cervical vagotomy is performed to cause pulmonary edema, decreased lung compliance, and atelectasis (BERRY et al., 1986). These effects also result in surfactant dysfunction (BERRY et al., 1986).

It is generally known that breathing high levels of oxygen ($FiO_2 > 0.9$) can result in tissue toxicity (KALLET and MATTHAY, 2013). Generally, high oxygen concentrations can injure all tissues of the body, but the lung is exposed directly to the highest partial pressure of inspired oxygen and thus is damaged the most (KALLET and MATTHAY, 2013). Here, severe hyperoxic acute lung injury (HALI) and alteration of surfactant function and metabolism can occur (DENEKE and FANBURG, 1980; KALLET and MATTHAY, 2013). The quandary of potentially giving rise to HALI during the treatment of ARDS by means of respiratory support can be leveraged in animal models for therapy on ARDS (NOTTER, 2000). It is notable that newborn animals have a higher resistance against oxygen toxicity than adult animals (DENEKE and FANBURG, 1980). Exogenous surfactant can help to improve pulmonary function in HALI (NOTTER, 2000).

A very common option for efficacy testing of exogenous surfactant is the use of lavage models. In lavage models, endogenous surfactant is either depleted by saline lavages or the lungs are excised and receive bronchoalveolar lavages afterwards

(NOTTER, 2000). For the performance of endogenous surfactant depletion, adult rabbits or rats are commonly used (ALBERT et al., 2009; ENHORNING et al., 1973; HAFNER et al., 1998b; HARTOG et al., 1997). A saline lavage model for adult sheep also exists (LEWIS et al., 1991). In lavage models autologous surfactant is washed out from fully developed lungs under anesthesia by performances of multiple saline lavages. This induces surfactant deficiency. After a certain surfactant deficient time, exogenous surfactant is administered and restoration of lung function is monitored. In this context, lung compliance and the recovery of oxygenation are assessed and evaluated especially. Rat lung lavage (RLL) trials are subject to several complications. For instance, test animals might decease before the end of trial. As a state of severe lung impairment is simulated in test animals, premature death may occur at all times during trials.

The advantage of the aforementioned whole animal models is the obtainment of detailed data on pulmonary function (NOTTER, 2000). However, substantial variations in respiratory data have to be accepted in test animals in these critical health conditions. Furthermore regarding clinical studies, conclusive results concerning new treatment concepts for ARDS are extremely challenging to attain, as physiological parameters in critically ill patients are difficult to control for (MATUTE-BELLO et al., 2008).

2.9.3. *Ex Vivo* Models

An *ex vivo* model of excised rat lungs was established in the 1970ies for efficacy testing of surfactant preparations to lower surface tension (BERMEL et al., 1984; HALL et al., 1992a; HOLM and NOTTER, 1987; IKEGAMI et al., 1977). In this model, lungs are excised carefully from adult rats while the heart is removed. They are then vacuum degassed while the lungs are submerged in saline (BERMEL et al., 1984). The lungs are then inflated with a PIP of 30 cmH_2O and slowly deflated to record a pressure-volume curve for baseline lung compliance evaluation (BERMEL et al.,

1984). Afterwards, 15 repetitive lavages are performed, using 10 ml saline. After this procedure, surfactant deficient pressure-volume curves are generated (BERMEL et al., 1984). This model can then be used for the administration of exogenous surfactant preparations which can be administered in nebulized form or installed intratracheally (IKEGAMI et al., 1977). The effectiveness of surfactant administration is evaluated by analysis of quasi-static compliance improvements at several time points via the generation of pressure-volume curves (BERMEL et al., 1984; IKEGAMI et al., 1977; NOTTER, 2000). The advantages of the use of lavaged, excised lungs is the reliable reproducibility of data and, according to Bermel et al., the lungs can be used for several surfactant testings by leaching previously administered exogenous lung surfactant by means of saline lavages (BERMEL et al., 1984).

A more sophisticated setup of excised rat lungs is the *ex vivo* model of isolated perfused rat lungs (IPL). This model allows the constant ventilation of excised lungs with positive or negative pressure settings and simultaneously perfusion of the organ is enabled. With this setting the maintenance of organ viability over several hours is enabled. Hence, in contrast to lavaged excised rat lungs in which only the biophysical effect of exogenous surfactant on surface tension lowering can be assessed, the IPL model allows the prediction of physiological impacts of exogenous surfactant preparations using *ex vivo* lungs.

2.10. Efficacy Testing of Surfactant Batches

Due to variations in the production of protein-containing products, regulatory agencies require the determination of activity in each production unit. Tests must be performed to ensure the stability and thus the consistent quality of every surfactant batch. Furthermore, the effectiveness of each exogenous surfactant batch is examined before the medication is authorized for administration to patients. Therefore, one of the aforementioned preterm or adult animal models is used. The *in vivo* models used for efficacy testing all lead to very severe conditions in the test animals, which can result in preterm decease before the end of the study. The most

important parameter in efficacy testing of surfactant batches is the improvement of oxygenation. Consequently, the intention is to achieve reduced breathing ability in test animals. The insufficient gas exchange may lead to hypoxemia and hypercapnia that can cause respiratory acidosis and systemic metabolic acidosis (BROUILLETTE and WAXMAN, 1997; RODRIGUEZ, 2003; WARLEY and GAIRDNER, 1962). These severe health conditions can lead to premature decease of the test animals. Moreover, procedures can lead to pulmonary edema formation. Edema fluid, which contains plasma proteins, leads to an inactivation of surfactant and thus is not wanted in efficacy testing (HOLM and NOTTER, 1987; NOTTER, 2000). Complete pulmonary edema can furthermore lead to impaired gas exchange and can also result in premature decease of test animals.

2.11. Aim of the Study

As not all test animals reach the end of surfactant efficacy trials, the aim of this study was to establish an *ex vivo* test model for efficacy testing of artificial lung surfactant batches.

Therefore, lungs and hearts were isolated as a bundle from rats. *Ex vivo*, the lungs were then ventilated and artificially perfused. A scheme for repetitive bronchoalveolar lavage performances was established in the isolated lungs in order to obtain an *ex vivo* secondary surfactant deficiency model. The setup of the surfactant deficiency model then resulted in impaired gas exchange. Respiratory parameters were analyzed synchronically to assess real-time lung function impairment. BALFs were analyzed biochemically to assess the impact of saline lavages on the cellular level. The *ex vivo* model of impaired lung function was then used for efficacy testing of surfactant formulations. The obtained data were compared to efficacy data from RLL trials. Therefore, one aim of the present undertaking was to assess whether pO_2 standard deviations in IPLs are smaller compared to RLL trials. By comparing these data, it was assessed if the *ex vivo* model of isolated perfused rat lungs was a suitable alternative for efficiency testing of surfactant batches. Furthermore, the *ex vivo* model was used to compare efficacies of different surfactant compositions. Thus, more insights about adequate surfactant formulations for the treatment of the Respiratory Distress Syndrome and the Acute Respiratory Distress Syndrome were gained.

3. Materials and Methods

3.1. Chemicals

96 well microplates	R&D Systems Europe, Ltd., Abingdon, UK
Albumin bovine Fraction V	SERVA Electrophoresis GmbH, Heidelberg, Germany
CONOXIA® GO_2X	Linde AG, Pullach, Germany
Control serum, HS 2611	Randox Laboratories Ltd., Crumlin, UK
Copper(II) sulfate pentahydrate	Merck KGaA, Darmstadt, Germany
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics GmbH, Mannheim, Germany
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific Inc., Waltham, MA, USA
Ethanol puriss. p.a., absolute, ≥99.8%	Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Eukitt® mounting medium	Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Folin-Ciocalteu's phenol reagent	Merck KGaA, Darmstadt, Germany
Isotone Kochsalz-Lösung 0.9%	B. Braun Ag, Melsungen, Germany
Ketamine 10% (ketamine hydrochlroride)	bela-pharm GmbH & Co. KG, Vechta, Germany
LIVE/DEAD ® Viability/Cytotoxicity Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
Narcoren®	Merial GmbH, Hallbergmoos, Germany
Natriumhydrogencarbonat 8.4% solution for infusion	B. Braun Ag, Melsungen, Germany
Pancuronium-Actavis	Actavis Group PTC ehf., Hafnarfjördur, Island
PBS	R&D Systems Europe, Ltd., Abingdon, UK
PBS Dulbecco	Biochrom GmbH, Berlin, Germany

Plate Sealers	R&D Systems Europe, Ltd., Abingdon, UK
pO_2 ZERO SOLUTION	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Potassium sodium tartrate tetrahydrate	Merck KGaA, Darmstadt, Germany
rat TNF-α ELISA Kit	R&D Systems Europe, Ltd., Abingdon, UK
Reagent Diluent	R&D Systems Europe, Ltd., Abingdon, UK
Richard-Allan Scientific™ Histoplast-Paraffin	Thermo Fisher Scientific Inc., Waltham, MA, USA
Rompun® 2% (xylazine hydrochloride)	Bayer Vital GmbH, Leverkusen, Germany
Rotipuran® (Formalin 37%)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium azide	Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Sodium carbonate, anhydrous	Merck KGaA, Darmstadt, Germany
Sodium hydroxide, pellets for analysis	Merck KGaA, Darmstadt, Germany
Stop Solution	R&D Systems Europe, Ltd., Abingdon, UK
Substrate Solution	R&D Systems Europe, Ltd., Abingdon, UK
Thermoklar®	BIOMED Labordiagnostik GmbH, Oberschleißheim, Germany
Triton® X-100	Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Universal-Agarose, peqGOLD	VWR International, Radnor, PA, USA
Wash Buffer	R&D Systems Europe, Ltd., Abingdon, UK

3.2. Material

Cannulae Set for Rat to Core System IPL-2	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Cobas Cuvettes	Globe Scientific Inc., Paramus, NJ, USA
Corning® Costar® cell culture plates 6 well, flat bottom	Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Filtropur BT 50 bottle top filter 500 ml, 0.22 µm pore size, 90 mm PES-membrane	Sarstedt AG & Co, Nürnbrecht, Germany
Imaris software	Bitplane AG, Zurich, Switzerland
Lockable one-way stopcock	Vygon, Ecouen, France
Makrolon® type IV cages	ZOONLAB GmbH, Castrop-Rauxel, Germany
Menzel-Gläser Superfrost microscope slides	Gerhard Menzel GmbH, Braunschweig, Germany
pO2 Zero Solution	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
R/M-H „V1534“ (rat feeding)	ssniff-Spezialdiäten GmbH, Soest, Germany
Sample Cups with Cap for Roche Cobas	Globe Scientific Inc., Paramus, NJ, USA
Shandon™ EZ Double Cytofunnel™	Thermo Fisher Scientific Inc., Waltham, MA, USA
Sterilium®	BODE Chemie GmbH, Hamburg, Germany
Three-way stopcock	Sarstedt AG & Co, Nürnbrecht, Germany

3.3. Devices

ATLANTIS Microplate Washer	Biochrom Ltd., Cambridge, UK
Axioskop 2 Plus	Carl Zeiss Microscopy GmbH, Jena Germany

Babylog 8000 Plus	Drägerwerk AG & Co. KGaA, Lübeck, Germany
Blood gas analyzer Type ABL 5	Radiometer Medical ApS, Brønshøj, Denmark
Blood Pressure Transducer P75	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Cobas Fara II centrifugal analyzer	Roche Diagnostics GmbH, Mannheim, Germany
Confocal Laser Scanning Microscope Meta 510	Carl Zeiss Microscopy GmbH, Jena, Germany
Consul Coverslipper	Fisher Scientific GmbH, Schwerte, Germany
CytoSpin III centrifuge	Thermo Fisher Scientific Inc., Waltham, MA, USA
CytoSpin III slide clamping devices	Thermo Fisher Scientific Inc., Waltham, MA, USA
EMM Electrometer Amplifier	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Epoch Microplate Spectrophotometer	BioTek Instruments, Inc., Winooski, VT, USA,
Flow Probe Type 2N	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
IPL-2 Core System 230 V 73-4275	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Krumdieck Tissue Slicer	TSE Systems GmbH, Bad Homburg vor der Höhe, Germany
Lung weight measurement to core system	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Microm™ Rotary microtome	Thermo Fisher Scientific Inc., Waltham, MA, USA
Microplate reader	Tecan Group Ltd., Männedorf, Switzerland
Mini Flow-Through CO_2 Electrode 1/16	Hugo Sachs Elektronik Harvard Apparatus

	GmbH, March-Hugstetten, Germany
Mini Flow-Through O_2 Electrode 1/16	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Mini Flow-Through pH Electrode 1/16	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Op. table size 5 for IPL 2	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
OPPM Oxygen Partial Pressure Measurement Amplifier	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
PH-electrode	Hamilton Bonaduz AG, Bonaduz, Switzerland
PHMM and pHCM Plugsys pH Measurement and Control Module type 694 and 694/1	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
pHMM pH Amplifier	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
PLUGSYS Basic System Case 603	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
PLUGSYS Case, Type 603	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Pneumotachometer PTM and Differential Low Pressure Transducer DLP 2.5, type 381	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
REGLO Digital MS-4/8 Reglo-Digital Roller Pump	IDEX Health & Science GmbH, Wertheim, Germany
Thermostatic circulator E 103, 3 l, 230 V/50 Hz	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Timer Controller Module Type 686	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany
Transit Time Flowmeter Module	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany

Varistain™ 24-4 Automatic Slide Stainer	Thermo Fisher Scientific Inc., Waltham, MA, USA
Ventilation Control Module Type 681	Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany

3.4. Statistical Analysis

Acquired respiratory data were analyzed and others were calculated with the PULMODYN® Pulmonary Mechanics Data Acquisition Software (*Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany*). All data were exported to GraphPad Prism® software (version 4.03) (*Graphpad Software Inc., La Jolla, CA, USA*). Within the raw data, incorrect measurements obtained e.g. during BAL performances were excluded from data evaluation. Statistical analysis was performed within GraphPad Prism® using Student's t-test or One-way ANOVA and Tukey's Multiple Comparison Test. Data in histograms is presented as means + SEM. A p-value ≤ 0.05 was considered statistically significant.

3.5. Test Animals and Test Item

Specific-pathogen-free, male Sprague-Dawley rats (Crl:CD (SD)), Charles River Laboratories International, Inc., Sulzfeld, Germany) were used for IPL trials. Animals weighed 272 ± 36.15 g and were kept under standard environmental conditions (20 ± 2 °C, 55 ± 15% relative air humidity, 12 h light-dark cycle) in groups of 3 to 4 in Makrolon® type IV cages in the animal husbandry of the Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM). Animals were fed with complete feedingstuff for rats and mice V1534 and water ad libitum (Stadtwerke Hannover AG, Hannover, Germany).

Organ dissections for IPL trials were executed in accordance with the German law on animal welfare (file number: 33.9-42502-05-10A100, Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany).

In RLL and IPL trials, aerosolized recombinant surfactant protein-C (rSP-C) was administered. RSP-C is a white, spray-dried powder with a mass median aerodynamic diameter (MMAD) of 1.7 µm (geometric standard deviation (GSD) 2.3) (POHLMANN et al., 2013).

A total of 16 IPLs was subdivided into groups of four. One group consisted of control IPLs that received bronchoalveolar lavages but did not get surfactant after the lung function was impaired. For surfactant efficacy testing, different surfactant mixtures were administered to the three test groups. Performance of dipalmitoyl-phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) assays for surfactant analytics were performed by PHAST Development GmbH & Co. KG, Konstanz, Germany and financed by Takeda GmbH, Singen, Germany (data not shown). Table 1 shows the content of recombinant surfactant protein C in relation to the standard rSP-C preparation in the tested surfactant batches:

Table 1: Exogenous Surfactant batches that were tested in IPLs.

Sample	Name	Relative rSP-C content
1	Placebo	0%
2	20% Surfactant	20.12%
3	100% Surfactant	100%

For surfactant efficacy testing the aerosol was generated at a flow rate of 1.14 l/min (1.0 l/min sheath flow + 0.14 l/min pulse air). 250 Puffs with a frequency of 10 Puffs/min were administered to the lung.

Filter samples were taken for the calculation of the administered surfactant dose. For this purpose, a critical nozzle with a flow rate of 0.22 ml was installed at the tracheal cannula suspension. The flow rate of 0.22 ml represented the V_T of repetitively

lavaged IPLs. Vacuum was connected to the critical nozzle and 20 puffs surfactant aerosol were pulled onto a filter.

3.6. Rat Lung Lavage (RLL) Model

RLL trials were conducted at Fraunhofer ITEM for routine efficacy testing of exogenous surfactant batches and were not part of the present thesis. The data of rat lung lavage experiments were used in this thesis to compare pO_2 levels before and after bronchoalveolar lavages and surfactant administration to IPL trials. Furthermore, lavage fluid was used for biochemical analysis and comparison to BALF from IPL trials. Within the comparative RLL-study, recombinant rSP-C was administered to a total of 16 male Sprague-Dawley rats (220 - 300 g).

The rat lung lavage trials complied with the current Standard Operating Procedures for the RLL model at Fraunhofer ITEM and were conducted according to the Principles of Good Laboratory Practice (German Chemicals Law §19a, Appendix 1, Federal Law Gazette pp. 3498-3532, August 28, 2013). The RLL trials were performed according to the regulations of the German Animal Protection Law of May 18, 2006.

Following the determination of pre-treatment blood gas levels, rats underwent repetitive BALs to induce a secondary surfactant deficiency. After the BAL performances were completed, aerosolized surfactant was administered and further analyses of blood gas levels were performed. By this procedure, the recovery of lung function was investigated and thus the efficacy of the given surfactant batch could be evaluated. In order to assess the efficacy of the applied surfactant aerosol, the oxygen saturation of the blood was evaluated in the form of oxygen partial pressure and lung compliance assessment. These levels were taken to represent the recovery of lung function.

In brief, for RLL trials, the test animals were anesthetized intravenously. Therefore, 0.10 ml Narcoren® were injected in the tail vein to rats with a body weight between 200 and 279 g, or 0.11 ml to test animals with a bodyweight > 280 g, respectively. An arterial catheter was then introduced into the carotid artery and a tubus was placed into the trachea. After the end of the dissection, 0.25 ml Pancuronium bromide (2 mg/ml) i.m. and 1.5 ml sodium hydrogen carbonate/glucose-solution i.p. were administered to the rats before connecting them to pressure-controlled positive ventilation. Test animals were ventilated with 100% medical oxygen (FiO_2 = 1.0), a breathing frequency of 35 breaths/min and an inspiration to expiration ratio (I:E) of 1:2. Positive peak inspiratory pressure (PIP) was set to 17 cmH_2O while PEEP was 3 cmH_2O during preparation. After all test animals were connected to the ventilator, PIP/PEEP was increased for approximately 10 min to 26/6 cmH_2O to obtain baseline values of blood gas analysis. During the following rest period of 15 min, the pressure was turned back to 17/3 cmH_2O. Before the start of BALs, PIP/PEEP was increased again to 26/6 cmH_2O until the end of the experiment.

Bronchoalveolar lavages were carried out using 37°C warm saline according to Table 2. In the beginning 4 BALs were carried out repetitively at 5 min intervals on all 4 test animals. If the partial arterial oxygen pressure was ≥ 180 mmHg, additional BALs were performed until pO_2 was < 180 mmHg.

Table 2: Saline volumes for BALs in RLL trials.

Body weight [g]	220-234	235-249	250-265	266-279	280-294	295-300
BAL volume [ml]	7.5	8.0	8.5	9.0	9.5	10.0

Blood samples were taken via the arterial catheter and analyzed for contents of pO_2, pCO_2, pH, saturation of blood with oxygen (sO_2), hydrogen carbonate (HCO_3^-) and actual base excess (ABE). On average, 8 samples were taken for analysis of blood gases at baseline after performance of 4 repetitive BALs and after every following BAL. Additionally, blood samples were taken 5, 30 and 60 min after the end of the last BAL performance in all test animals and 10 and 60 min after surfactant

administration. Test animals exhibiting bloody BALFs were excluded from the analysis.

At 60 minutes after the last BAL performance, aerosolized surfactant was administered to the test animals. Humidified rSP-C surfactant aerosol was generated using a similar constructed CPA and humidifier as in IPL trials. The settings of the CPA in RLL trials were: 10 pulses/min, surfactant aerosolization of approx. 15-20 mg/min, 15 min delivery duration, and an aerosol flow rate of 1.14 l/min (1.0 l/min sheath flow + 0.14 l/min pulse air). This resulted in a concentration of 13.2 to 17.5 mg/l administered over 15 min. After surfactant administration, the breathing frequency was reduced in three steps of 5 breaths/min every 10 min to 20 breaths/minute.

Blood samples were taken and recovery of pO_2 was analyzed at 10 and 60 min after surfactant treatment. At the end of an experimental day, the test animals were detached from the ventilation system and euthanized with an arterial injection of 0.2 ml Narcoren®. After dissection of the carcasses, judgments about pulmonary edema formation and lung ventilation status were made. Furthermore, pressure controlled ventilation measurements were carried out to assess static lung compliance. In order to evaluate the efficacy of a tested surfactant batch, at least 10-12 rats had to meet the following criteria:

Baseline: $pO_2 > 400$ mmHg
End of BALs: $pO_2 < 180$ mmHg
60 min after last BAL, before surfactant administration: $pO_2 < 110$ mmHg

A surfactant batch was classified as efficient for restoration of lung function if the average pO_2 level 10 or 60 min after treatment was ≥ 400 mmHg.

Results from an exemplary RLL trial (n = 16 male Spargue-Dawley rats) were compared to results from IPL trials using a different surfactant batch with the same composition.

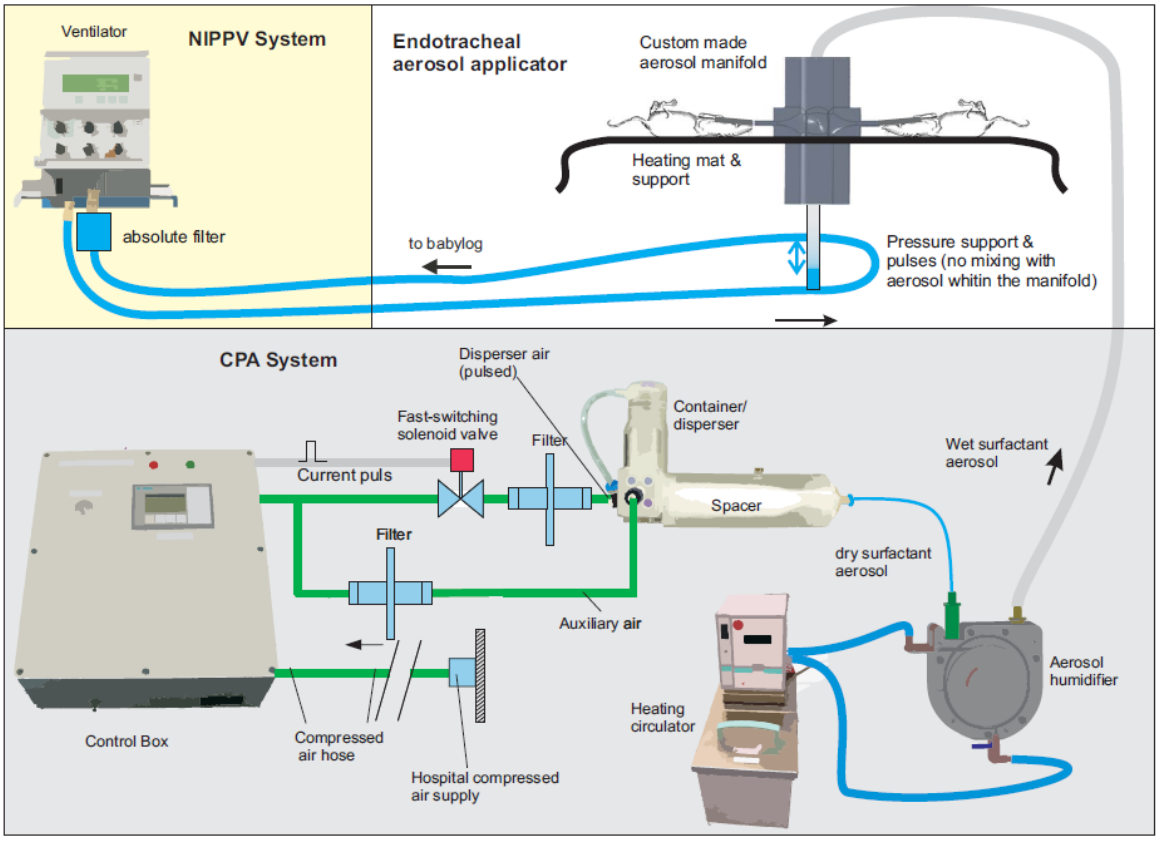

Figure 1: RLL setup for testing of aerosolized surfactant batches. Rats were ventilated in a supine position using a nasal intermittent positive pressure ventilation (NIPPV) System. Exogenous surfactant was administered with the Continuous Powder Aerosolization (CPA) system.

3.7. Isolated Perfused Lung Setup

In order to refine surfactant batch efficacy testing *in vivo*, an *ex vivo* perfusion system for rat lungs was used. The isolated perfused rat lung (IPL) model is a commercially available system and is used in different setups. We used an IPL apparatus size 2 core system (*Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany*) which can be used for the ventilation and perfusion of rat or guinea-pig lungs. The core system consists of a glass chamber which represents an artificial thorax and allows negative pressure ventilation. This chamber can be mounted to a

Plexiglas® baseplate which provides a mobile chamber lid with openings for the ventilation and the perfusion of the organ.

For the adjustment of different settings and monitoring of parameters, several modules are connected to the core system. These modules are gathered in PLUGSYS system cases which are connected to a computer for further computing of respiratory parameters with the PULMODYN® software and monitoring of lung function. Every 4 s, all parameters are measured or calculated. For instance, for a trial of 240 min, 3600 data points are acquired.

For controlling respiratory parameters, a ventilation control module was integrated in the IPL system. This module allows a switch from positive to negative pressure ventilation and also enables ventilation with breathing frequencies between 30 and 100 breaths/min, I:E set between 10 and 90%, and adjustments of end-inspiratory pressure (EIP) between - and -26 cmH_2O and end-expiratory pressure (EEP) between -1 and -3.6 cmH_2O. To solve slow lung collapse, deep inspiration (DI) breaths are performed physiologically in humans and animals. To mimic this behavior, a timer controller module was integrated in the PLUGSYS system which allows the performance of DI breaths in certain time intervals.

A pneumotachometer was connected to the chamber lid of the artificial thoracic chamber, when the lung was negatively ventilated. This allowed the direct data acquisition of the inspiratory and expiratory flow which was then used for the calculation of V_T, R_L and C_{dyn}. The chamber lid also includes a weight measurement unit. Lung weight data were acquired with an edema balance module in the PLUGSYS system which allowed an online assessment of edema or atelectatic formations.

The isolated organ could be perfused with blood or with an artificial buffer, while perfusion could be under constant flow or constant pressure. For constant flow perfusion, the flow rate of buffer through the isolated organ was set by a roller pump. For constant pressure perfusion, the perfusion pressure was determined by the hydrostatic pressure in the lung’s arterial supply line. For correct measurement of the

perfusion pressure, the pressure transmitter had to be on the exact same level as the pulmonary vein. The flow rate of perfusion was measured with a flow probe and transferred to a flowmeter module. Perfusion flow data were recorded with the PULMODYN® software.

For animal sparing perfusion, an artificial Krebs-Henseleit-buffer was used and prepared fresh before every trial.

- Krebs-Henseleit-buffer:

  NaCl 6.896 g/l (118 mmol/l)

  KCl 0.351 g/l (4.7 mmol/l)

  $NaHCO_3$ 2.092 g/l (24.9 mmol/l)

  KH_2PO_4 0.163 g/l (1.2 mmol/l)

  $MgSO_4$ x 7 H_2O 0.296 g/l (1.2 mmol/l)

  Glucose 1.00 g/l (5.55 mmol/l)

  $CaCl_2$ 0.368 g/l (3.32 mmol/l)

  BSA 4% 40 g/l

After sterile filtration through a filter of 0.2 µm pore size, Aminoplasmal® 1.100 g/l (22 mmol/l) was added. Before donor dissection, buffer was transferred into a reservoir using a roller pump. A pH meter was inserted to the reservoir and connected to a pHMM PLUGSYS pH measurement module in order to monitor the buffer's pH. Strict observation of the pH is crucial and pH should remain at 7.35 ± 0.04 to ensure the functionality of the isolated organ. Therefore, a CO_2-regulator was connected to a pHCM PLUGSYS pH control module. If the buffer was too alkaline, CO_2 was released automatically into the buffer throughout the whole trial. PH data were acquired throughout the trial with the PULMODYN® software. From the reservoir, the buffer was drawn by a second roller pump through a bubble chamber and afterwards through the arterial and venous cannulas of the perfusion system. The perfusion of the lung with constant pressure leads to an increase in intravascular pressure (P_i) which in turn causes transmural vascular pressure (P_t) to rise according to the following equation:

$$P_t = (P_i + P_e) - P_a$$

Equation 5

This effect was even further amplified through negative pressure ventilation which leads to a reduction in extravascular pressure (P_a) in the artificial thoracic chamber. P_e represents the pressure in a pressure balancing chamber that was integrated in the perfusion circuit to lower P_i and prevent perfusate being drawn into the thoracic chamber during negative pressure increase.

At the same position, two pressure transducers were installed which recorded the pressure in the pulmonary artery (PAP) and in the left atrium (VP). It is important to ensure that PAP does not exceed 12 cmH_2O, as high PAP quickly leads to lung edema formation.

The perfusate system was also used for blood gas analysis. For this purpose, a junction was integrated in the tubing system which allowed drawing perfusate from the perfusion system to electrodes for analysis of partial oxygen pressure (pO_2), partial CO_2 pressure (pCO_2) or pH in the perfusion system. Using a three-way valve, blood gases could be analyzed in the perfusate before or after lung entry.

Calibration of pO_2 was performed at 0 mmHg with pO_2 zero solution and at 145 mmHg using compressed air gassed water at 37°C.

A thermostatic circulator bath was connected to the buffer reservoir and the negative pressure chamber in order to keep the lung at a physiological temperature of 37°C. Thermoklar® was added to prevent algal growth in the thermostatic circuit.

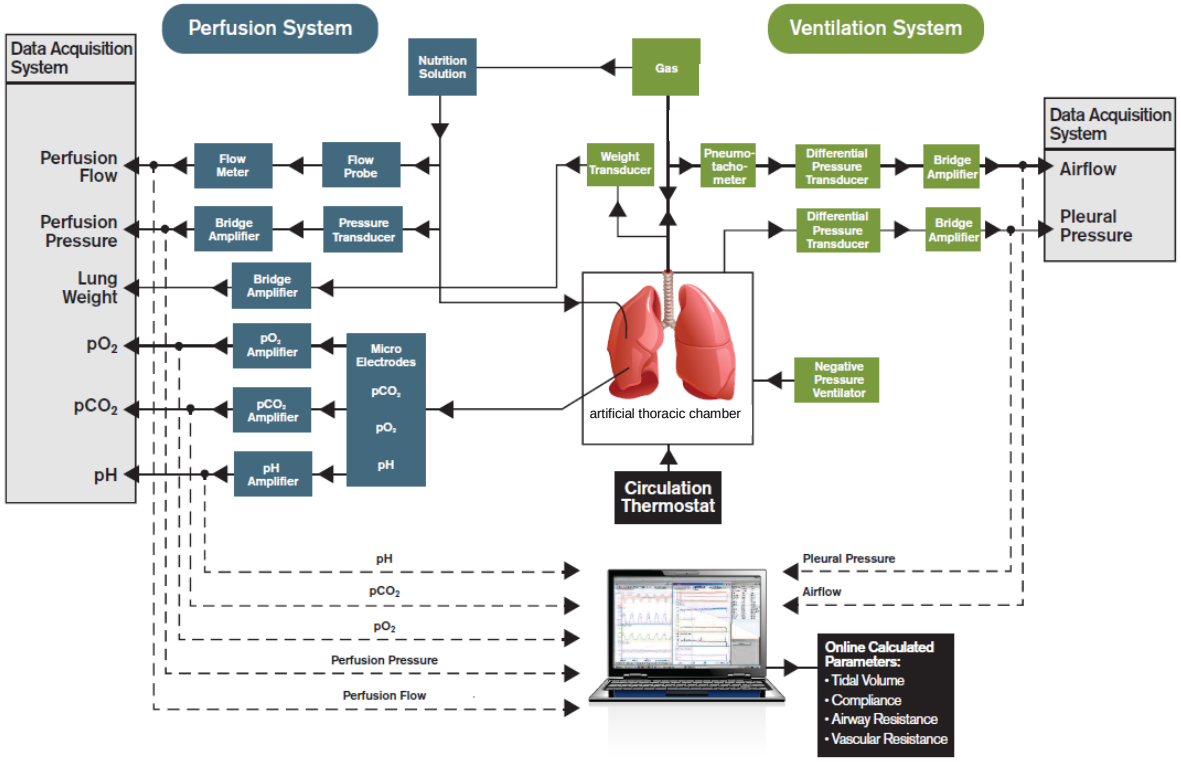

Figure 2: Block diagram of the IPL system. Modified from Hugo Sachs Elektronik - Harvard Apparatus GmbH. Used with permission.

3.7.1. Dissection and Preparation of Lungs

Before dissection, ketamine hydrochloride (70 mg/kg bw) and Rompun® 2% (xylazine hydrochloride 6 mg/kg bw) were injected intraperitoneally into male Sprague-Dawley rats. Deep anesthesia was verified with the absence of response to interdigital toe pinch. After the fur was moisturized with Sterilium® and water, the test animal was fixed in supine position on a operating table adjacent to the IPL system. The surgical table was mounted to same height in which the isolated lung was placed in IPL's chamber later on. Thus, the risk of hydrostatic edema formation was reduced.

At the start of dissection, an incision in the trachea was made. A cut through a hyaline membrane was made and a tracheal cannula for the IPL-2 system (length:

17 mm, outer diameter 2.5 mm, inner diameter 1.95 mm) was inserted into the trachea which was secured with a ligation. The tracheal cannula was then connected to the chamber lid of the IPL system and with this to positive pressure ventilation (7.5 cmH_2O, frequency: 80 min-1, I:E: 50:50) resulting in a V_T of approximately 1.5 ml (UHLIG and TAYLOR, 1998).

A median laparotomy along the costal arches was performed and the diaphragm was excised. To avoid thrombi formation, heparin (2000 IE/ kg BW) was injected into the ventriculus cordis sinister. After the anticoagulant had distributed in the blood circulation (30 s), the donor animals were exsanguinated with a cut into the vena cava.

With a parasternal section, the Cavum thoracis was opened and the ribs were clamped to the surgical table. The thymus was removed and a ligature was placed under the truncus pulmonalis. An incision was then made in the left ventricle to avoid an increase in arterial pressure after insertion of the arterial cannula. Before performing an incision into the right ventricle, absence of air bubbles in the perfusion system was checked, to avoid air embolism when inserting the arterial cannula. The arterial cannula was then inserted into the right ventricle and pushed through the pulmonary semilunar valve into the pulmonary trunk. After placing a ligature around the heart, the venous cannula was pushed from the left ventricle through the mitral valve into the left atrium. Via this procedure, a closed circuit was established which allowed constant flow perfusion of the isolated lung with Krebs-Henseleit buffer with a flow of approx. 5 ml/min.

While ensuring continued perfusion and ventilation, the lung-heart convolute was removed from the thoracic cavity. The isolated lung was still connected to the chamber lid of the IPL system and with this mounted into the chamber of the IPL system. The chamber was then tightly closed and ventilation was switched to negative pressure (EIP/EEP -7.5/-3 cmH_2O, frequency: 80 min-1, I:E: 50:50) while perfusion was switched from constant flow to constant pressure. This resulted in a mean flow rate of 19 ± 3.32 ml/min. If PAP was above 12 cmH_2O, ventilation pressure was switched again to positive pressure, the artificial thoracic chamber was

removed and the positions of the arterial and venous cannulae were corrected. A pneumotachometer was then mounted into the chamber lid and online recording of data initiated. At the same time, (DI) breaths with an EIP of -20 cmH_2O were started, to be carried out every 5 min to solve lung adhesions which occur physiologically.

3.8. Surfactant Deficiency Model

For testing the efficacy of surfactant batches, a surfactant deficiency model was established using the IPL system. After an equilibration time of 30 min, lungs were ventilated with medical oxygen (FiO_2 = 1.0). Ventilation with 100% oxygen allowed a maximum measuring range of pO_2 during bronchoalveolar lavage performance as well as precise assessment of pO_2 increase after administration of surfactant. PO_2 values in the perfusate were measured throughout every IPL trial. For this purpose, perfusate was withdrawn from the perfusion system right behind the venous cannula. With a relatively slow flow of 10 ml/min electrodes for gas analysis were supplied with the perfusate. Within 20 min after medical oxygen supply, a plateau for pO_2 values was reached.

After 50 min EIP was lowered from -7.5 to -15 cmH_2O, while EEP remained at -3.6 cmH_2O. This setting was used to enhance the lung tissue's viability after repetitive BALs were performed.

After an adjustment period of 5 min to an EIP of -15 cmH_2O, at the time point 56 min, the first bronchoalveolar lavage was performed 1 min after DI execution. For every BAL performance, 8 ml of warm (37°C) saline were used. The artificial thoracic chamber was opened which stopped the lungs breathing ability immediately and resulted in a lack of record of respiratory parameters for the time of BAL performance. The tracheal cannula was disconnected from the chamber lid and a syringe containing warm saline was connected to the tracheal cannula. The saline was slowly pushed into the lung tissue and was gently withdrawn immediately afterwards. The tracheal cannula was mounted to the chamber lid and the artificial

thoracic chamber was closed again. A single BAL was performed in less than 1 min. The lung was able to breathe right after closure of the chamber and respiratory parameters were monitored again. The process of V_T, pO_2, and lung weight were especially monitored prior to the performance of a BAL. If V_T was ≤ 0.4 ml, execution of DI and BAL were terminated and pO_2 was monitored. When pO_2 was < 180 mmHg, a 1 h period of surfactant deficiency was started (Figure 1).

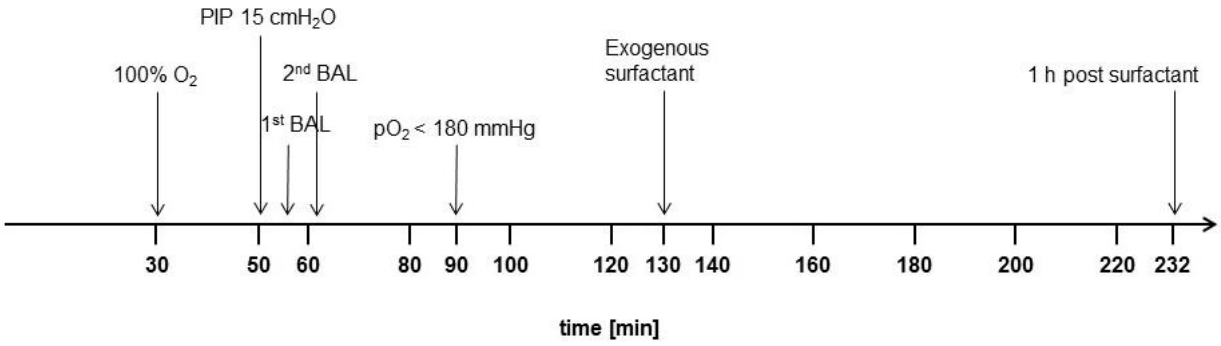

Figure 3: Scheme for surfactant efficacy testing in IPLs.

3.9. Exogenous Surfactant Administration

Testing of artificial lung surfactant was started 60 min after pO_2 dropped < 180 mmHg. For surfactant administration, a continuous powder aerosolizer (CPA) was used, which allows aerosolization of dry surfactant powders (POHLMANN et al., 2013). The CPA system consisted of different parts. Up to 3 g of surfactant powder were filled into a powder reservoir, which was assembled together with a disperser/aerosolizer and a spacer in a container-disperser-spacer (CDS). Pressurized medical oxygen was provided to the CDS by a supply unit. Transient pulses of pressurized oxygen interacted with surfactant powder which was released from a reservoir into the dispersion zone. The releasing frequency therefore determined the mass flux and the volumetric flow rate of aerosol. Surfactant aerosol was then transported, while heated, and humidified in the aerosol conditioning/humidification device. Subsequently, humidified surfactant aerosol was

transferred to a wet aerosol delivery tube (WADT) in which it was cooled down to 37°C before being delivered to the lung (Figure 4) (POHLMANN et al., 2013). The WADT was connected to the ventilation system of the IPL set-up by removal of the pneumotachograph. For optimal aerosol supply to the lung tissue, the original ILP-2 system was modified. In the original set up, surfactant administration was insufficient because IPLs had to overcome a dead volume to inhale exogenous surfactant from the aerosol stream. This dead volume was composed of the volume of the WADT-adapter of 0.98 ml and the volume of the tracheal cannula of 0.44 ml, yielding a total of 1.42 ml. However, lavaged IPLs have a reduced V_T of 0.21 ml (

Table 4). After insertion of an adapter that enabled aerosol supply directly to the tracheal cannula, only the tracheal cannulas dead volume had to be overcome by the breathing lung.

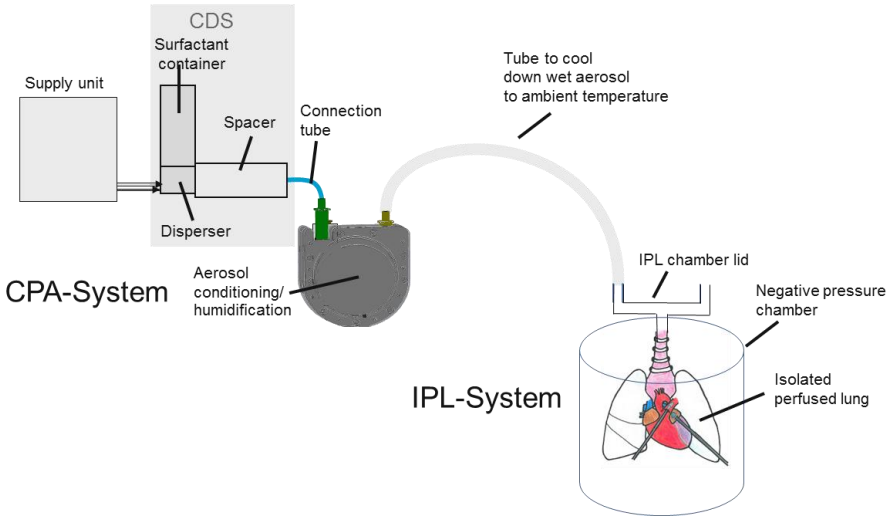

Figure 4: Connection of CPA-System to IPL-System.

3.10. Lung Tissue Analysis

The viability of the lung tissue after performances of repetitive BALs in IPLs was assessed by use of the two diagnostic methods LIVE/DEAD and Hematoxylin and eosin (HE) staining. Moreover, cytospots were performed to analyze the cell recruitment ability of the *ex vivo* organ.

3.10.1. LIVE/DEAD staining

The LIVE/DEAD Viability/Cytotoxicity Kit was used for staining. The kit's procedure consists of a two-color fluorescence cell viability assay that is based on the determination of live and dead cells by means of intracellular esterase activity and plasma membrane integrity detection. Live cells are visualized by intracellular esterase activity. Non-fluorescent, cell-permeable calcein acetoxymethylester is converted to fluorescent calcein, which is retained in live cells, producing green fluorescence. Ethidium homodimer-1 (EthD-1) enters cells through damaged membranes and binds to nucleic acids. As a result, red fluorescence is emitted by dead cells, while EthD-1 is excluded by intact plasma membranes of live cells.

At the end of trials, IPLs were filled with 1.5% agarose in Dulbecco's Modified Eagle Medium. Tissue slices with a thickness of 400 µm were prepared using a Krumdieck tissue slicer and placed into 6-well plates. The components of the LIVE/DEAD Viability/Cytotoxicity Kit were diluted to a staining concentration of 4 µm in PBS. 300 µl of the staining solution were added to each well. The plate was covered to avoid direct light exposure and incubated for 24 h. Three washing steps with PBS were performed before the slices were viewed under a confocal microscope. Fluorescent calcein was measured at a wavelength of 495 nm (emission 515 nm),

while EthD-1 was measured at 495 nm (emission 635 nm). A scanning depth of 30 µm was used.

3.10.2. H&E staining

H&E stains are based on the blue coloration of nuclei by hemalum, which is a complex formed by the oxidation product of haematoxylin, i.e. hematein, and aluminium ions. After nuclear staining, eosin Y solution is added. This entails counterstaining of eosinophilic structures which will then appear colored red. The sample preparation and examination of histological slides was performed by colleagues of the pathologic department of Fraunhofer ITEM. IPLs from control trials were chosen for H&E stains. These lungs were exposed to repetitive BALs, but no surfactant was administered. At the end of the control trial, IPLs were immersed in buffered 4% formalin solution for at least 24 h. Lungs were then divided into individual lung lobes, dehydrated in 70% ethanol and coated with paraffin wax. Tissue slices with a thickness of 3 - 4 µm were produced using a rotary microtome. Subsequently, coloring with haematoxylin and eosin Y was performed using a tissue stainer. A coverslipper was used for covering the samples with Eukitt® and coverslips. The stained lung slices were evaluated by a toxicopathologist using a bright-field microscope.

3.10.3. Cytospots

Cytospots were taken from bronchoalveolar lavage fluids. Therefore, 1 ml aliquots from single BALs were put on ice. After the end of the IPL trial, the cell concentration in the lavage fluid was counted. If the cell concentration was higher than 80.000 cells / ml, BALF was diluted to a cell concentration of 80.000 cells / ml with saline solution to a total volume of 200 µl. This volume was used for every Cytospot. After slides

were labeled and Cytofunnel were merged with a clamping device, they were inserted into the holding spaces of the centrifuge. The samples were centrifuged for 10 min at 1500 rpm. From each sample, two Cytospots were made, using Double Cytofunnel slides. The final preparations are qualitatively assessed by visual inspection, dried and stained by Pappenheims method. Therefore, slides were fixed in May Gruenwald solution for 5 min and then rinsed with tap water. For the following Giemsa staining, Giemsa stock solution was diluted 1:20 with tap water. The resulting staining solution was stable for about one hour and had to be prepared freshly for each staining. After a 10 min staining period with Giemsa solution, the slides were again rinsed gently with tap water and were allowed to dry. Cells were then analyzed under 40x- magnification objective.

3.11. Biochemical Analysis of Bronchoalveolar Lavage Fluid

The potentially harmful effect of the ventilation setting in the IPL model for surfactant efficacy testing as well as the impact of saline BALs on the lung tissue was investigated biochemically using the lactat dehydrogenase (LDH)-Assay, measuring of total protein and determination of the interleukin TNF-α.

3.11.1. Lactate Dehydrogenase Assay

The cytotoxic impact of saline BAL was determined by measuring cytoplasmatic lactat dehydrogenase (LDH). LDH is expressed in every cell. Under physiological conditions, LDH catalyzes the reduction of pyruvate to lactat by oxidation of $NADH/H^+$ to NAD^+. This reaction provides oxidated NAD+ for glycolysis. LDH is of great importance to the organism as it is also a catalyst for the reverse reaction of lactat oxidation to pyruvate by the reduction of NAD^+ to $NADH/H^+$. An increase in LDH in the test matrix indicates an impact on cell membrane integrity. By use of

$NADH/H^+$, the FAD-binding flavoprotein diaphorase in the assay mixture is reduced to $FADH_2$. In a second step, $FADH_2$ then reduces the assay's dye solution 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) to formazan which can be measured photometrically. An increase of LDH in the test matrix correlates directly to the amount of formed formazan.

For the performance of the assay, aliquots from the first five bronchoalveolar fluids from IPL and RLL trials were collected. Additionally, at the end of a trial a final BAL was performed in both setups and a further aliquot was collected.

For positive control, a bolus instillation of 500 µl Triton-X-100 for cell lysis was incubated for 5 min in IPLs at the end of a trial. Afterwards, BAL was performed and an aliquot was collected. All aliquots for LDH analysis were put on ice immediately.

Right before assay performance, the reaction mixture was prepared by mixing the catalyst (diaphorase / NAD^+ mixture) with the dye solution (INT and sodium lactat). A 96-well plate was filled with duplicates of 100 µl/well of every BAL-aliquot. Then, 100 µl reaction mixture was added to every well and the plate was incubated with protection from light for 30 min. Using a microplate reader, the absorbance of the samples was measured at a wavelength of 492 nm while the background signal was measured at 630 nm. In order to relate the cytotoxicity to the positive control percentage-wise, the background signal was subtracted from the sample signal and was then divided by the positive control's signal and multiplied by 100 (Equation 6).

$$\text{cytotoxicity} = \frac{(\text{sample signal at 492 nm-background signal at 630 nm})}{\text{Triton-X-100}} * 100$$

Equation 6: Calculation of cytotoxicity from LDH assay

3.11.2. Total Protein Assay

Protein measurement was conducted to assess the impact of repeated saline BALs and the ventilation scheme on the lung tissue in the IPL model. If the cell's

membrane function is impaired, intracellular proteins are released into the cellular surroundings. Protein concentrations were measured by Lowry protein assay, using a Cobas Fara analyzer. This method is based on the formation of a copper-complex in the presence of peptide bonds and the reduction of phosphotungstic acid and phosphomolybdic acid by aromatic amino acids. The formed copper complex can be measured colorimetrically.

The complete BALF of the consecutively performed BALs 1 through 5 was collected. At the end of trials, an additional final BAL was performed and BALF was collected. All samples were immediately cooled to -20°C and stored at -80°C till protein analysis. The following solutions were prepared beforehand:

- Protein standard stock solution (1 g/l):
  50 mg Albumin bovine Fraction V and 50 mg sodium azide was dispersed in 50 ml double distilled water (ddH_2O)
- Sodium hydroxide solution (0.1 mol/l):
  4.0 g sodium hydroxide pellets was dispersed in 1000 ml ddH_2O
- Potassium sodium tartrate solution:
  10 g Sodium carbonate and 0.1 g potassium sodium tartrate tetrahydrate are dispersed in sodium hydroxide solution (0.1 mol/l)
- Copper(II) sulfate solution:
  50 mg copper (II) sulfate pentahydrate was dispersed in 10 ml ddH_2O

For quantification of protein samples, dilutions of a daily prepared standard stock solution were measured in a calibration rack before analyzing BALF samples. To this end, the following dilutions were mixed and put into the calibration rack:
0.050 g/l = 25 µl stock solution + 475 µl ddH_2O
0.100 g/l = 50 µl stock solution + 450 µl ddH_2O
0.200 g/l = 100 µl stock solution + 400 µl ddH_2O
0.300 g/l = 150 µl stock solution + 350 µl ddH_2O
0.400 g/l = 200 µl stock solution+ 300 µl ddH_2O

Before running each protein analysis, a calibration with the aforementioned dilutions was performed and quality control measurements (purchased control serum and control serum in a 1 to 300 dilution in ddH_2O) were taken.

The following two solutions were prepared right before total protein measurement:

- Solution A:
  1.0 ml Folin-Ciocalteu's phenol reagent dispersed in 1.8 ml ddH_2O
- Solution B:
  10 ml potassium sodium tartrate solution was mixed under stirring with copper (II) sulfate solution

Solution A and B were put into the first two positions of the analyzer's sample rack. For mixing the sample and the reagent solutions, Cobas cuvettes were placed into the analyzer. Samples of 100 to 500 µl of the first five BALFs and the final BALF were directly pipetted into Cobas sample cups and placed into the sample rack. The analyzer was heated up to 37°C and 20 µl of each sample was pipetted into a separate Cobas cuvette. After that, the analyzer mixed the sample with 200 µl of solution B for an incubation period. To start the reaction, 20 µl of solution A were pipetted into the same cuvette. The absorbance of formed copper-complexes was then measured at a wavelength of 750 nm. The samples were then quantified with the calibration curve. If a sample contained high concentration of protein (>0.4 g/l), the sample was diluted in ddH_2O and analyzed again.

3.11.3. TNF-α Assay

TNF-α was analyzed using a sandwich ELISA for rat TNF-α. The following solutions were needed:

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtered

Wash buffer: 0.05% Tween® 20 in PBS, pH 7.2 - 7.4

Reagent diluent: 1% BSA in PBS, pH 7.2 - 7.4, 0.2 µm filtered

Substrate solution: 1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine)

Stop solution: 2 N H_2SO_4

For the preparation of a 96-well plate, mouse anti-rat TNF-α capture antibody was diluted in PBS to a working concentration of 4 µg/ml. The microplate was immediately coated with 100 µl per well of capture antibody. The plate was then sealed and incubated overnight. Each well was aspirated and washed with wash buffer. This process was repeated twice. Using a microplate washer, each well was washed three times with 400 µl wash buffer. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. The reaction in the 96-well plate was then blocked by adding 300 µl of reagent diluent to each well. The plate was subsequently incubated for one hour. Again, each well was aspirated and washed with wash buffer. This process was repeated twice. Using the microplate washer, each well was washed three times with 400 µl wash buffer. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Per well, 100 µl of every BALF, of BALF diluted 1:2, or recombinant rat TNF-α standard were added. The standard was reconstituted with 0.5 ml of reagent diluent. A seven point standard curve using 2-fold serial dilutions was produced. The plate was covered with an adhesive strip and incubated for two hours. After the rest period, each well was aspirated and washed with wash buffer. This process was repeated twice. Using the microplate washer, each well was washed three times with 400 µl wash buffer. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. To each well 100 µl of reconstituted biotinylated goat anti-rat TNF-α detection antibody in 1 ml dilution reagent was added. The plate was then covered with an adhesive strip and incubated for two hours. The washing and aspiration process was repeated hereafter. Each well was aspirated and washed with wash buffer. This process was repeated two times. Using the microplate washer, each well was washed three times with 400 µl wash buffer. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels.

To each well, 100 µl of the working dilution of streptavidin conjugated to horseradish-peroxidase were added. The plate was covered and incubated for 20 min in the dark. Again, each well was aspirated and washed with wash buffer. This process was repeated two times. Using the microplate washer, each well was washed three times with 400 µl wash buffer. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Substrate solution (100 µl) was added to each well. The plate was incubated for 20 min in the dark. To each well 50 µl of stop solution were added. The plate was gently tapped to ensure thorough mixing. Immediately thereafter, the optical density of each well was determined at 450 nm, using a microplate reader set to 450 nm. The wavelength correction was set to 570 nm. The readings at 570 nm were subtracted from the readings at 450 nm. Data were analyzed using a 4 point curve fitting.

4. Results

4.1. IPL setup for Bronchoalveolar Lavages

4.1.1. Standard IPL Setting

Before setting up a scheme for the provision of *ex vivo* bronchoalveolar lavages, normal IPL values were recorded. For this, IPLs were set up with a ventilation pressure ratio of EIP/EEP of -7.5/-3.0 cmH_2O, a ratio of inspiration to expiration of 1:1, a breathing frequency of 80 breaths/min and a constant pressure perfusion, which resulted in a perfusion flow of 24.09 ml/min (SD 1.405). The mean tidal volume was 2.23 ml (SD 0.11), while the mean increase in lung weight after a study time of 200 min accounted to 424.5 mg (SD 154.26) (Figure 5).

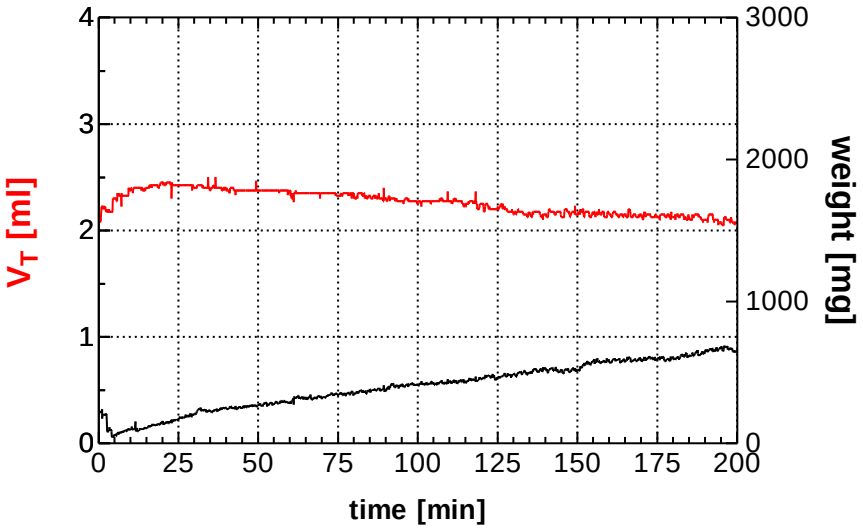

Figure 5: V_T and weight process. Mean V_T and lung weight in standard IPL settings are shown. Values are given as means, n = 4.

4.1.2. End-Inspiratory Adjustments

Different end-inspiratory pressure (EIP) settings were tested in the beginning of the IPL setup for the use as a secondary surfactant deficiency model. The aim was to find the highest possible EIP setting which would prevent the alveoli from collapsing after bronchoalveolar lavage performance without resulting in edema formation. The respiratory parameter V_T was analyzed to assess the impact of high EIP on the respiratory flow. Lung weight was analyzed to display the onset of edema formation. End-expiratory pressure (EEP) was set to -3.6 cmH_2O as this was the lowest feasible setting in the IPL-2 system. An EIP of -15 cmH_2O led to an increase in weight of 671 mg, while an EIP of -17 cmH_2O resulted in a weight gain of over 1000 mg within 180 min. An EIP of -20 cmH_2O led to a weight gain of over 1000 mg within 20 min (Figure 6). An EIP of -26 cmH_2O led to an immediate exponential weight gain (Figure 7). Furthermore, V_T was affected by lower EIP settings. An EIP of -15 cmH_2O as well as an EIP of -17 cmH_2O led to a slow linear decrease in V_T while an EIP of -26 cmH_2O resulted in an exponential decrease in V_T after 75 min.

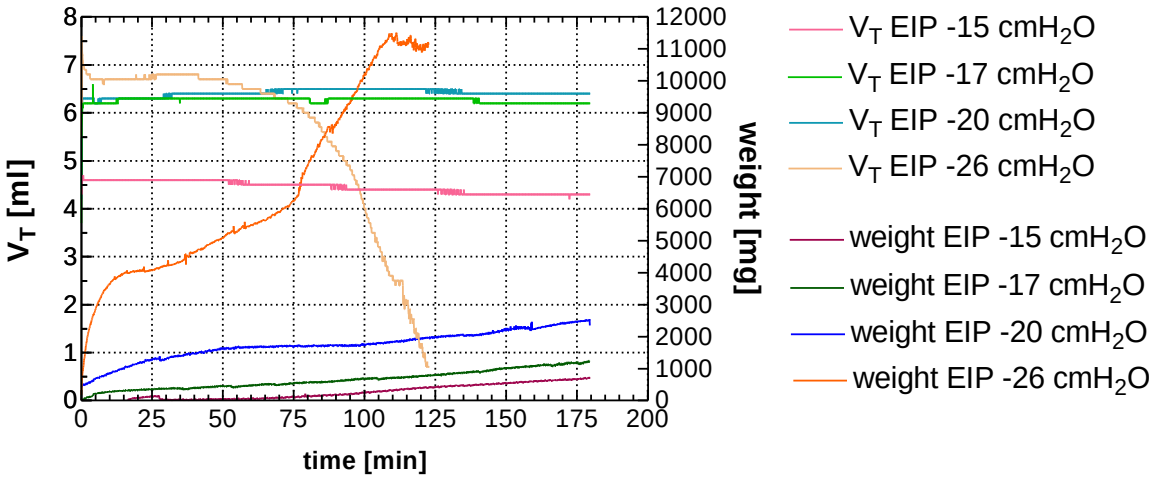

Figure 6: V_T and weight process with EIP settings. Different EIP settings were tested in IPLs with an EEP of -3.6 cmH_2O. V_T and lung weight were measured, n = 1 for every EIP setting.

Figure 7: Edema formation in IPL. Negative ventilation pressure of EIP/EEP -26/-3.6 cmH_2O led to edema formations in IPLs. Artificial buffer in non-ventilated edematous lung tissue (brownish parts). Normal ventilated lung tissue (white parts).

4.1.3. Lavages

For bronchoalveolar lavage performances, 8 ml warm (37°C) saline was used. To assess the full impact of a single BAL to pO_2 levels, intervals of 30 min were set between lavage performances. With this procedure, it took around 105 min until pO_2 declined below 180 mmHg, which was the starting point for the period of impaired lung function of 60 min (Figure 8). To achieve a shorter time period for the acquisition of impaired lung function, the interval between the single BALs was reduced to 5 min. With this procedure, on an average, pO_2 was below 180 mmHg after 95 min after the beginning of a trial (Figure 9).

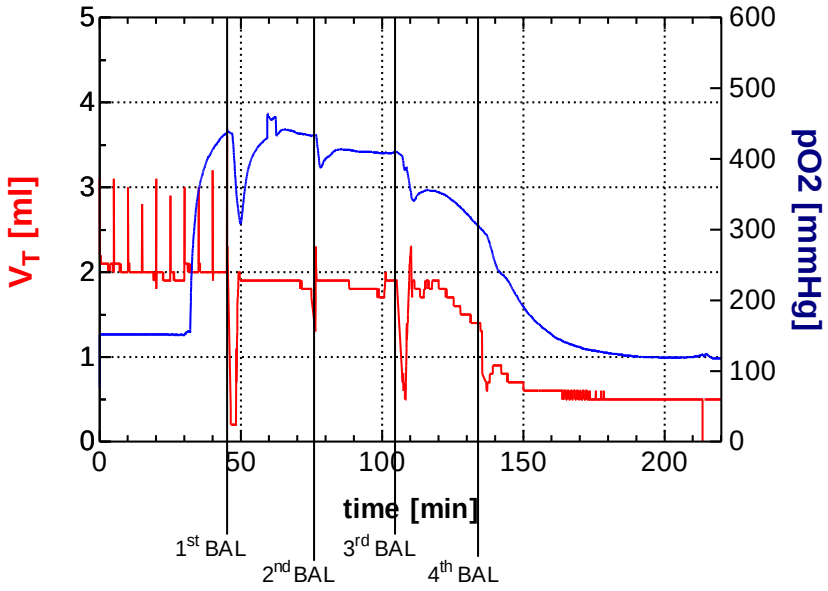

Figure 8: Examplary, V_T and pO_2 during BALs. A time interval of 30 min was left between single saline lavages, n = 1.

4.1.4. Respiratory Parameters

To assess the influence of BALs on lung physiology, oxygenation levels, as well as the influence to the respiratory parameters V_T, C_{dyn}, R_L were analyzed.

4.1.5. Oxygenation

A mean of 6 BALs (± 2.33, n = 16) led to a decrease in pO_2 of 322.5 mmHg (± 42.5, n = 16) within one hour of completion of the last BAL. A V_T of ≤ 0.4 ml was defined as a cutoff for BAL performance. When this status of reduced breathing ability was reached, a further decrease in pO_2 ensued even after BALs had been stopped. When pO_2 was below 180 mmHg, the time was recorded, allowing for a waiting period of 1 h of reduced breathing ability before starting artificial surfactant administration (Figure 9).

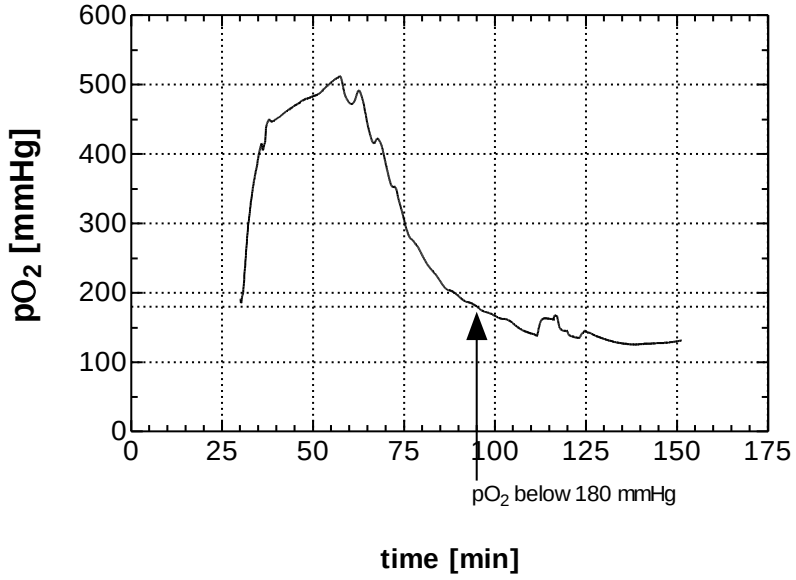

Figure 9: pO_2 in BAL. PO_2 in ventilated rat IPLs. Repetitive BALs were performed every 5 min, n = 16 lungs

For assessment of the impact of every single BAL to blood gas exchange, pO_2 values were analyzed every 5 min after a BAL. This time-shift analysis was needed as it took 2 min for the buffer to reach the electrodes for blood gas analysis. Additionally, the electrodes needed time to adjust for correct measurement at varying blood gas levels. Performance of a single BAL did not result in a significant difference in pO_2 (Figure 10).

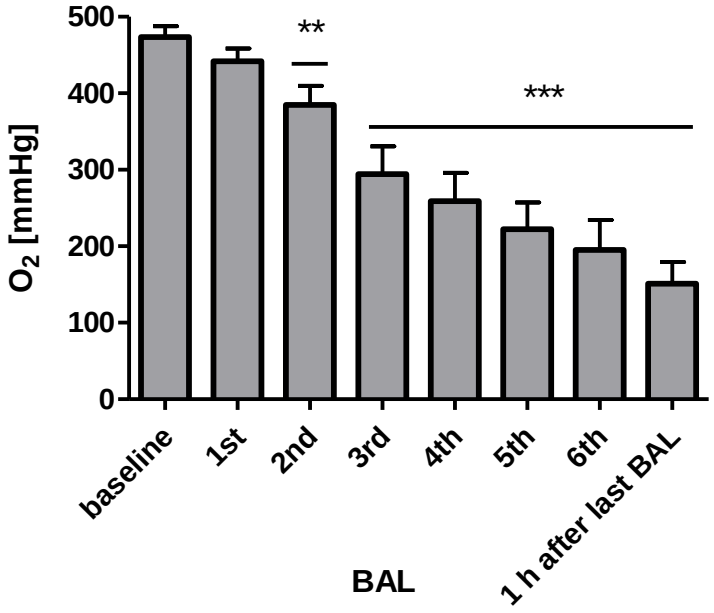

Figure 10: pO_2 levels after BAL performances. Repetitive BALs were performed every 5 min. Data were analyzed with two-tailed Student's t-test. Values are given as means +SEM, ** $p < 0.01$, *** $p < 0.001$ compared to baseline. Baseline to 2nd BAL and 1 h after last BAL: n = 16, 3rd BAL: n = 15, 4th BAL: n = 13, 5th BAL: n = 12, 6th BAL: n = 5

A second BAL led to a significant decrease in pO_2 of 18.77% ($p = 0.0039$) (Table 3). 6 BALs led to a decrease in pO_2 of 41.2% and oxygen levels reached a mean minimum of 151 mmHg 1 h after the last BAL was performed (Table 3) which was the point at which artificial surfactant administration began.

Table 3: PO_2 levels after 1st to 6th BAL.

pO_2	baseline	1st	2nd	3rd	4th	5th	6th	1 h after last BAL
Mean [mmHg]	473	442	384	294	259	222	195	151
SEM	13.93	16.30	24.78	36.15	37.12	34.78	39.36	28.21
%	100.00	93.33	81.23	62.17	54.70	46.95	41.17	31.92
IPLs (n)	16	16	16	15	13	12	5	16

4.1.6. Tidal Volume

V_T was analyzed to assess the influence of saline BALs on the inspired and expired gas volume. After equilibration time and administration of medical oxygen, EIP was set to -15 cmH_2O which resulted in a tidal volume of 3.79 ml (± 0.15). Performance of a single BAL led to slightly impaired breathing capability. V_T was reduced by 26.08% to 2.8 ml on average. A mean of 6 BALs (± 2.5) was needed to achieve a drop in tidal volume to ≤ 0.4 ml (Table 4).

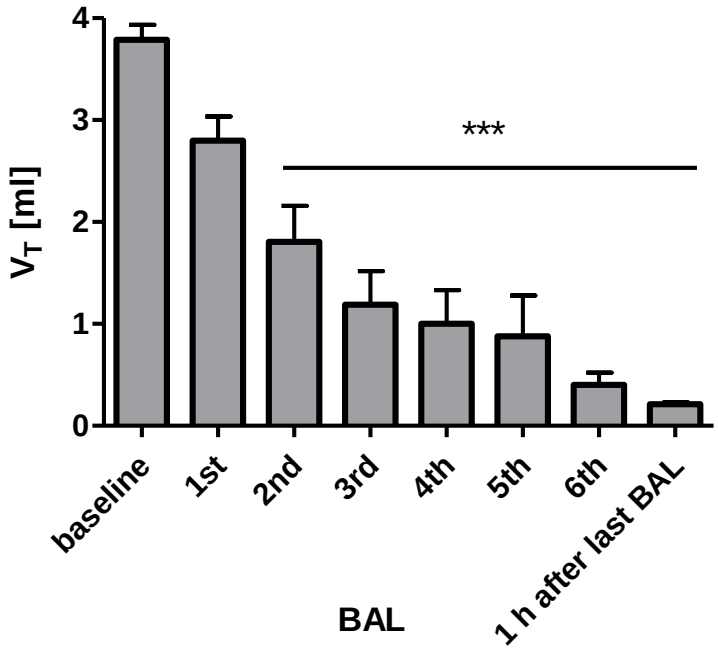

Figure 11: V_T levels after BALs (1 – 6). Repetitive BALs were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. Values are given as means +SEM, *** $p < 0.001$ compared to baseline. Baseline to 2nd BAL and 1 h after last BAL: n = 16, 3rd BAL: n = 15, 4th BAL: n = 13, 5th BAL: n = 12, 6th BAL: n = 5

Table 4: V_T levels after BAL performances. Loss of V_T after repeated BAL in IPLs.

V_T	baseline	1st	2nd	3rd	4th	5th	6th	1 h after last BAL
Mean [ml]	3.79	2.80	1.81	1.19	1.0	0.89	0.40	0.21
SEM	0.15	0.24	0.35	0.33	0.33	0.40	0.12	0.02
%	100	73.92	47.68	31.36	26.40	23.17	10.56	5.61
IPLs (n)	16	16	16	15	13	12	5	16

4.1.7. Dynamic Lung Compliance

C_{dyn} provides information about the extensibility of the lung tissue during active gas flow. The trials showed that C_{dyn} decreased over time and was improved again after performance of a deep inspiration breath with an EIP of 20 cmH_2O (Figure 12). Before repeated BALs were performed, EIP was lowered from -7.5 to -15 cmH_2O while EEP remained at -3.6 cmH_2O. This increase in inspiratory pressure lowered C_{dyn} by 17% (Table 5). A significant decrease in C_{dyn} was found after performance of a single BAL (Figure 13). Every BAL provoked a further decrease in C_{dyn}, whereas the performance of single DI breaths (20 cmH_2O) before every BAL caused C_{dyn} to nearly recover almost to the level before the BAL performance (Figure 12). After performance of 5 repeated BALs, C_{dyn} was almost completely impaired, at 12% of its baseline value.

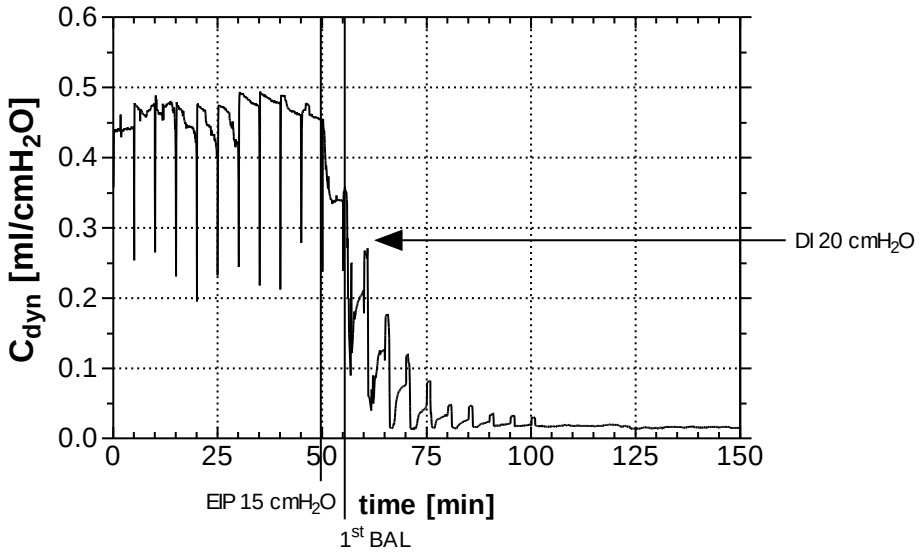

Figure 12: C_{dyn} during BALs. Repetitive BALs were performed every 5 min. Values are given as means, n = 16.

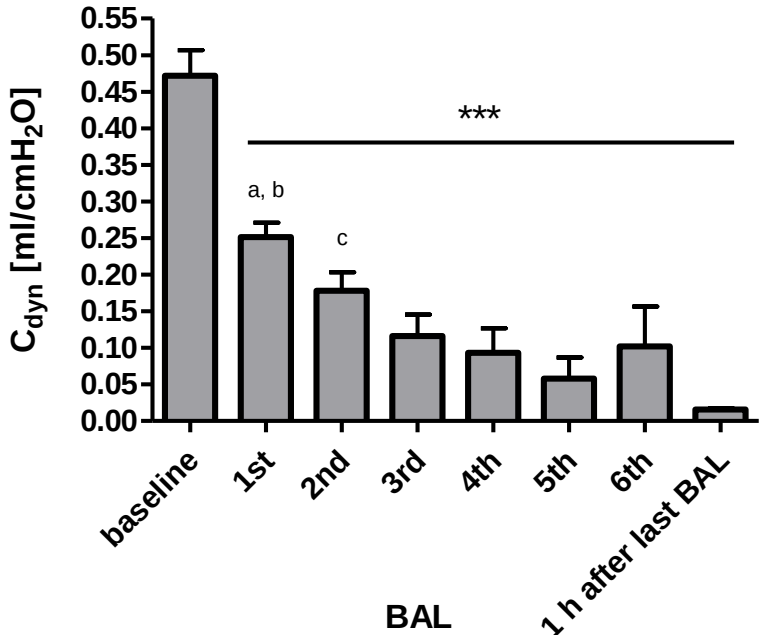

Figure 13: C_{dyn} during BALs. Repetitive BALs were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. Values are given as means, *** $p < 0.001$ compared to baseline, [a] $p < 0.01$ vs. 5th and [b] $p < 0.05$ vs. 3rd, [c] $p < 0.01$ vs. 1 h after last BAL,. Baseline to 2nd BAL and 1 h after last BAL: n = 16, 3rd BAL: n = 15, 4th BAL: n = 13, 5th BAL: n = 12, 6th BAL: n = 5

Table 5: C_{dyn} during BALs. Loss of C_{dyn} after repeated BAL in IPLs.

C_{dyn}	Baseline	1st	2nd	3rd	4th	5th	6th	1 h after last BAL
Mean [ml/cmH_2O]	0.47	0.25	0.18	0.12	0.09	0.06	0.10	0.02
SEM	0.04	0.02	0.03	0.03	0.03	0.03	0.05	0.00
%	100.00	53.26	37.72	24.58	19.78	12.33	21.62	3.33
IPLs (n)	16	16	16	15	13	12	5	16

4.1.8. Lung Resistance

In order to assess the influence of saline BALs on the bronchial airways, R_L was calculated. Performance of the 1st and 2nd BAL did not result in any increase of R_L. A significant increase in R_L was found after performance of the 3rd BAL (Figure 15) Furthermore, performance of a 4th, 5th, and 6th BAL resulted in a slight, albeit no significant, increase in R_L. One hour after performance of the last BAL, pulmonary resistance was found to be slightly below baseline level (Table 6).

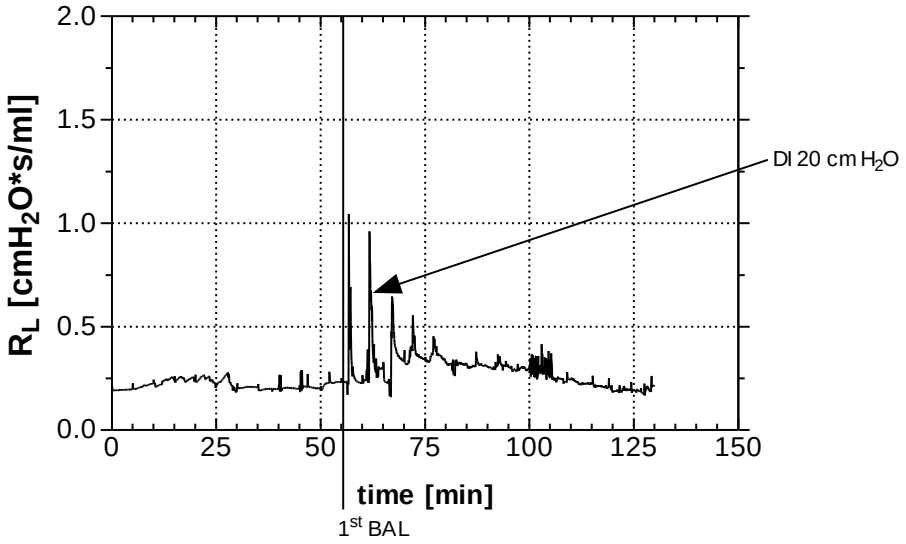

Figure 14: R_L during BALs. Repetitive BALs were performed every 5 min. Values are given as means, n = 16.

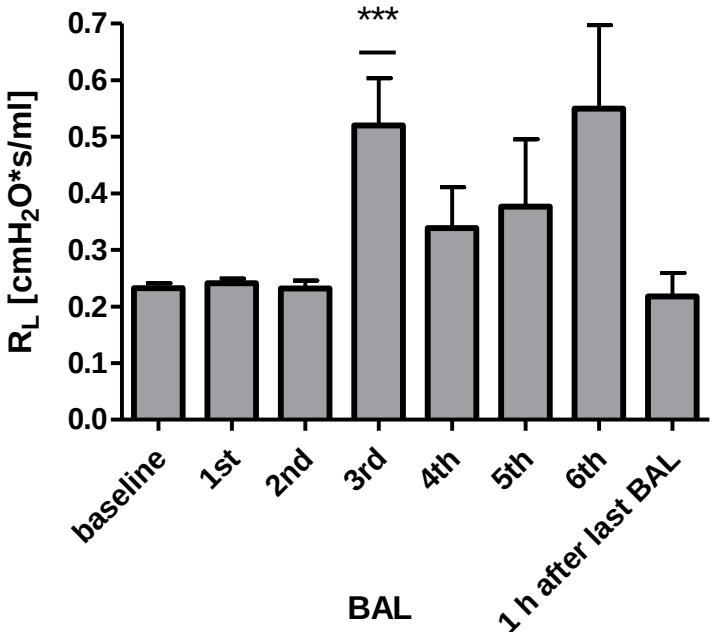

Figure 15: R_L during BALs. Repetitive BALs were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. Values are given as means +SEM, *** $p < 0.001$ compared to baseline. Baseline to 2nd BAL and 1 h after last BAL: n = 16, 3rd BAL: n = 15, 4th BAL: n = 13, 5th BAL: n = 12, 6th BAL: n = 5.

Table 6: R_L. Process of R_L during repeated BAL performance in IPLs.

R_L	Baseline	1st	2nd	3rd	4th	5th	6th	1 h after last BAL
Mean [cmH_2O*s/ml]	0.23	0.24	0.23	0.52	0.34	0.38	0.55	0.22
SEM	0.01	0.01	0.01	0.08	0.07	0.12	0.15	0.04
%	100	103.83	99.74	223.66	145.72	162.02	236.56	93.81
n	16	16	16	15	13	12	5	16

4.1.9. Lung Weight

Constant measurement of lung weight allowed a possible onset of edema formation in IPLs to be determined. The 2nd BAL led to an increase in lung weight of 327 mg, whereas succeeding BALs caused a mean weight gain of 30 mg (Table 7). No exponential increase in lung weight was found during and after BAL performances (Figure 16).

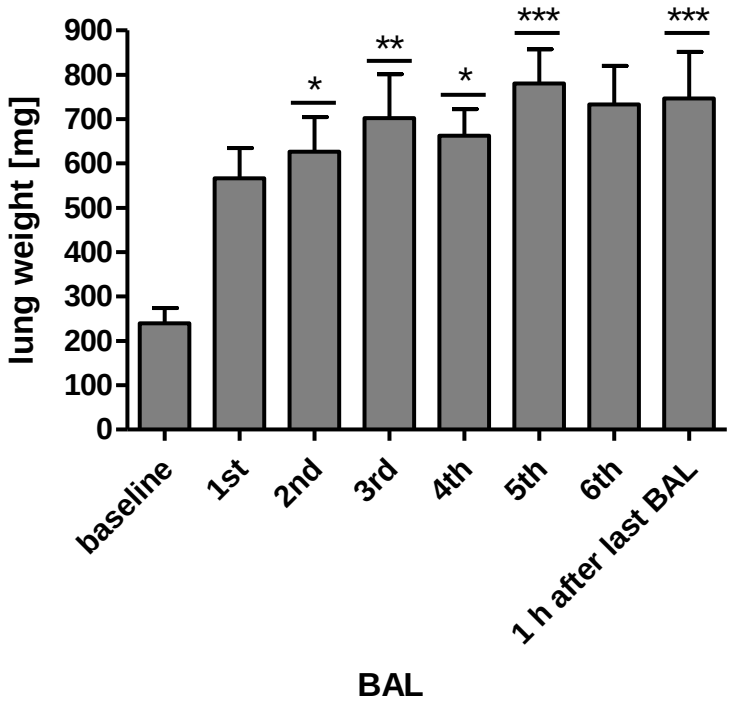

Figure 16: Lung weight during BALs. Repetitive BALs were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. Values are given as means +SEM, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to baseline. Baseline to 2nd BAL and 1 h after last BAL: n = 16, 3rd BAL: n = 15, 4th BAL: n = 13, 5th BAL: n = 12, 6th BAL: n = 5.

Table 7: Lung weight during BALs. Process of lung weight during repeated BAL performance in IPLs.

weight	baseline	1st	2nd	3rd	4th	5th	6th	1 h after last BAL
Mean [mg]	239.4	566.4	626.4	702.5	662.6	780.3	733.2	746.6
SEM	34.58	68.51	78.18	98.7	59.7	77.27	87.2	104.8
rel. weight gain from BAL [mg]		327	60	76.1	-39.9	117.7	-47.1	13.4
n	16	16	16	15	13	12	5	16

4.1.10. Lung Tissue Analysis

The impact of BALs on lung tissue was analyzed using LIVE/DEAD and HE stains.

LIVE/DEAD stains of mechanically ventilated and multiply lavaged IPLs showed viable lung tissue in yellow and dead cells in red (Figure 17).

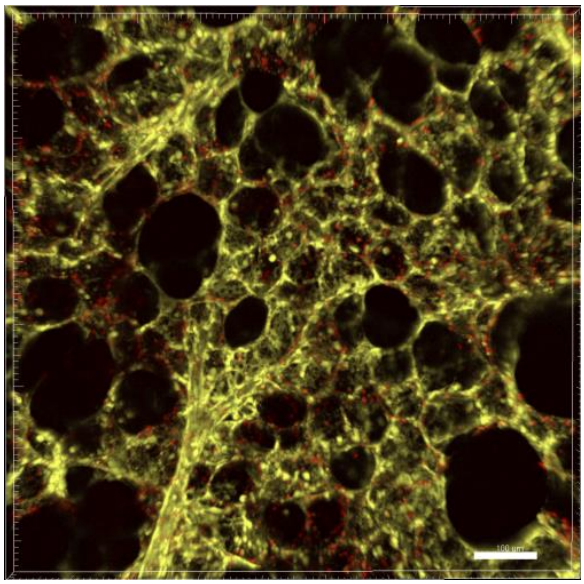

Figure 17: LIVE/DEAD staining of a lavaged IPL. Representative lung tissue slice from a mechanically ventilated IPL which was exposed to repetitive saline BALs (n = 7). (x100)

H&E stains of repetitively lavaged IPLs showed an extension of alveolar spaces. Some interalveolar spaces seem compressed while other alveolar spaces seem extended.

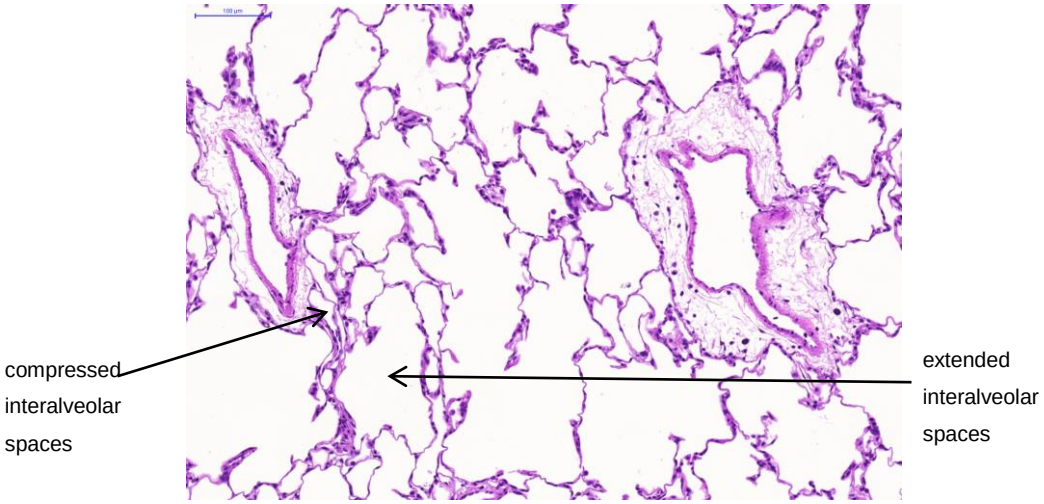

Figure 18: H&E staining of a lavaged IPL. Representative lung tissue from an IPL that was ventilated with a PIP/EEP of 15/3.6 cmH_2O for 240 min and was exposed to repetitive saline BALs.4. (x40)

4.2. Bronchoalveolar Lavage Fluid Analysis

4.2.1. Cell Analysis of BALFs

The cell content of bronchoalveolar fluids from repetitive BAL performances was analyzed. After performance of a single lavage, a high density of macrophages was found in the bronchoalveolar lavage fluid. Also, the presence of neutrophils and erythrocytes was noticed (Figure 19, Picture 1). The performance of a subsequent bronchoalveolar lavage after a 5 min interval showed high amounts of cellular debris (Figure 19, Picture 2). Cytospots were also taken after the end of trial. Therefore, the normal lavage scheme with repetitive saline lavages was performed and the IPLs were ventilated afterwards for at least 145 min. This time interval represented the 1 h- period of surfactant deficiency plus the time of surfactant administration and the 1 h-post surfactant administration time. Afterwards, the additional BAL was performed. Post-surfactant administration cytospots showed macrophages in the bronchoalveolar lavage fluid (Figure 19, Picture 3). As a positive control for lung tissue damaging interventions, Triton-X was administered to IPLs before performing

the additional BAL at the end of trial. The cytospots from positive control bronchoalveolar lavage fluid showed high contents of cellular debris and no intact cells (Figure 19, Picture 4).

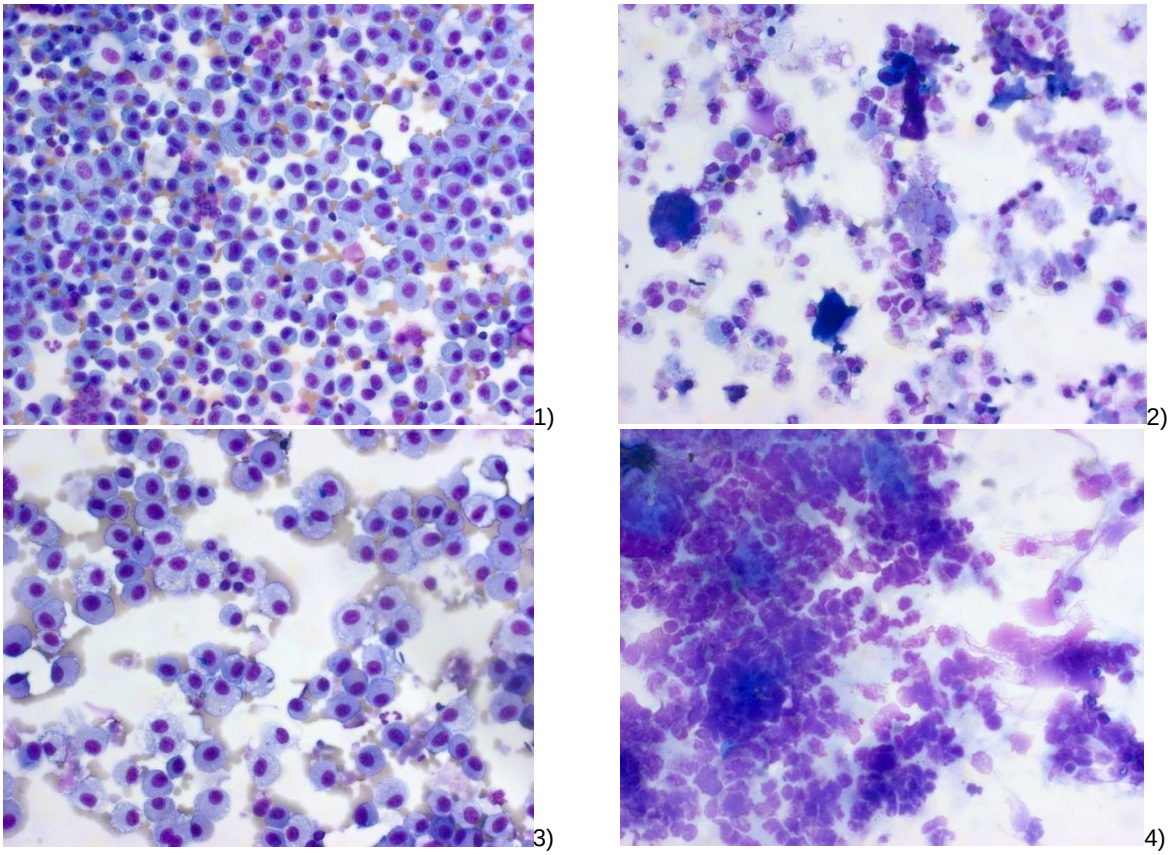

Figure 19: Cytospot of BALFs from an IPL. The IPL was ventilated with a EIP/EEP of -15/-3.6 cmH_2O for 240 min and was exposed to six repetitive saline BALs (x40). 1) 1st BALF, 2) 2nd BALF, 3) End-BALF after 240 min ventilation 4) Positive control: BALF after lung treatment with 1% Triton-X.

4.2.2. Lactat Dehydrogenase Analysis

LDH was measured in bronchoalveolar lavage fluid of IPL and RLL trials. Warm (37°C) saline bronchoalveolar lavages were performed consecutively in 5 min intervals in both lung models. The colorimetric measurement showed no increase in cytotoxicity for the performance of repeated saline BALs in both lavage models

(Figure 20). Moreover, no difference in LDH secretion level for the first four lavages between the IPL and RLL could be registered (Figure 20). In IPL trials, a scheduled fifth BAL was performed 5 min after the fourth BAL whereas the optional fifth BAL in RLL trials was performed 10 min after the fourth one. No significant difference could be found in LDH levels of the optionally performed fifth lavage between both models. In RLL trials, a significant difference in LDH could be found after surfactant administration at the end of trials, compared to LDH levels in regularly performed bronchoalveolar lavages.

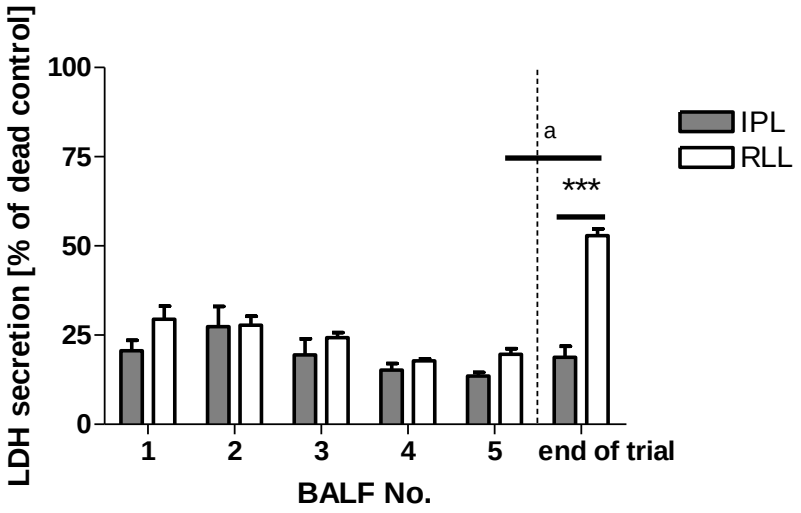

Figure 20: LDH secretion levels in BALF. Repetitive lavages were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. All values are given as mean + SEM, a $p < 0.001$ compared to BALF no. 1 to 5 from RLL trials, *** $p < 0.001$ compared between both models, n = 4 for each group.

4.2.3. Total Protein Analysis

The protein content in bronchoalveolar lavage fluids did not differ between single BALs. In comparison with the first BALF, a slight though not significant decrease in protein content could be seen ascertained for the second BALF. The whole protein content in the BALF obtained by saline lavage after the end of IPL trials, showed

significantly high protein contents compared to the bronchoalveolar lavage fluids from the repetitive lavage performance scheme (Figure 21).

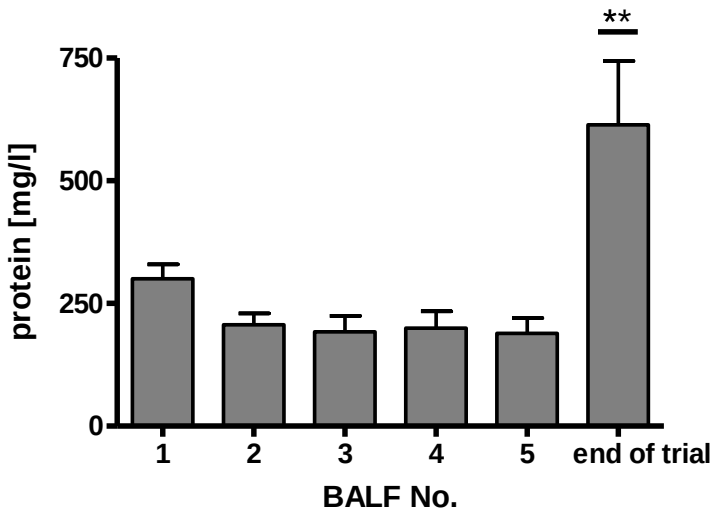

Figure 21: Total protein in BALFs from IPL trials. Total protein was determined in BALF from ventilated rat lungs in the IPL model. Repetitive BALs were performed every 5 min. Data were analyzed by one-way ANOVA and Tukey's Multiple Comparison Test. All values are given as mean + SEM, **p < 0.01 compared to BALF No. 1 to 5, n ≥ 4 for every sample.

4.2.4. TNF-α Analysis

TNF-α was measured to assess the impact of repetitive BALs and the ventilation settings on the acute inflammation response.

In BALs from repetitive BAL performances in RLL and IPL trials, no TNF-α was detected (data not shown). A significant difference ($p = 0.0423$) arose in BALFs after the end of RLL and IPL trials. A large standard deviation was found as in 2 out of 4 BALFs from RLL trials no TNF-α could be detected. Moreover, BALFs from IPL trials exhibited a large variation in TNF-α concentrations, ranging from 206.27 to 3553.14 pg/ml. The mean TNF-α concentration was 1189 pg/ml (± 309.3) in BALFs from IPL trials, and 49 pg/ml (± 89.62) in BALFs from RLL trials, respectively.

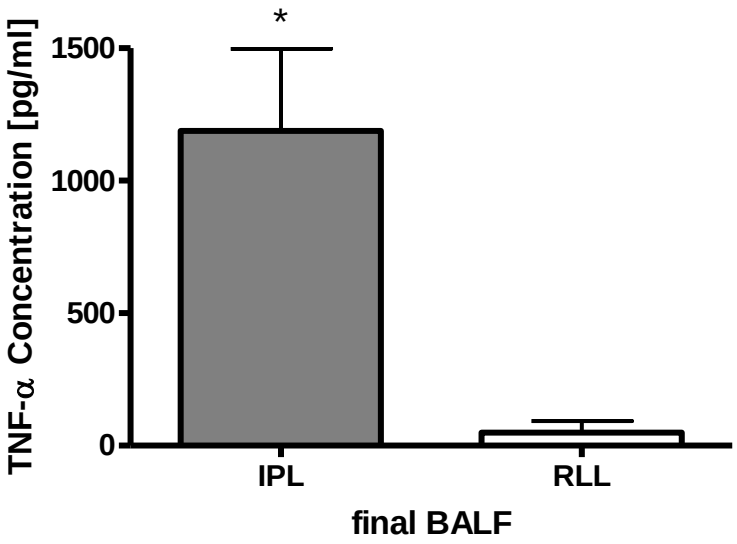

Figure 22: TNF-α in end-BALFs from IPL and RLL trials. Repetitive BALs were performed every 5 min. Surfactant was administered (150 puffs in RLL trials and 250 puffs in IPL trials, respectively). Data were analyzed by Student t-test. All values are given as mean + SEM, *p < 0.05 (n = 10 for IPL trials, n = 4 for RLL trials).

4.3. Exogenous Surfactant Administration in Lavaged IPLs

For the administration of exogenous surfactant to IPLs, the continuous powder aerosolization system was connected to the IPL-ventilation system. Warm, humidified rSP-C aerosol was supplied to the tracheal cannula that was connected to the trachea of IPLs. In the original setup the *ex vivo* administration of pulmonary surfactant did not result in an increase in tidal volume or partial oxygen pressure in IPLs with unchanged ventilation settings (Figure 23).

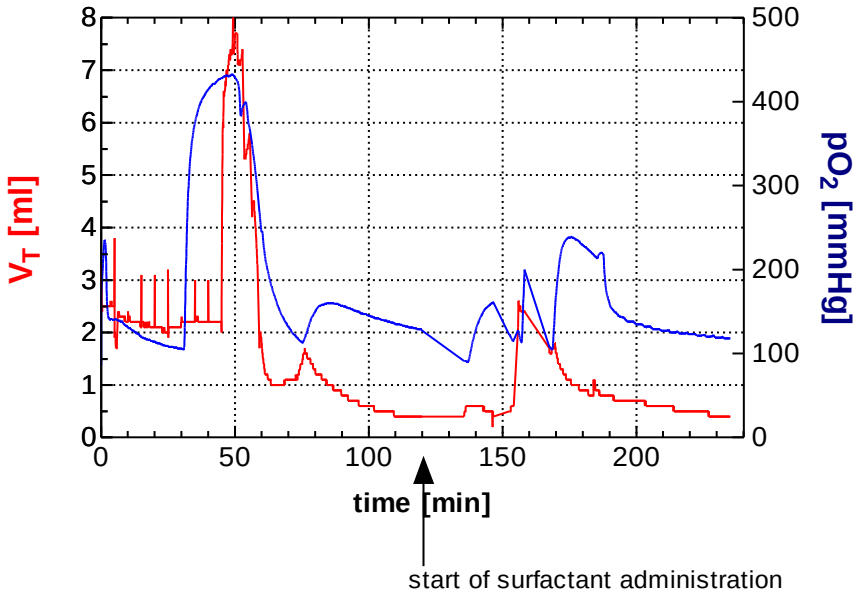

Figure 23: V_T and pO_2 during surfactant administration with standard ventilation setting. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered, n = 1.

In order to improve the oxygenation status during and after artificial surfactant administration, the end-inspiratory pressure was declined to -20 cmH_2O in the beginning of the administration interval. During surfactant aerosol administration 20 deep inspiration breathes with an EIP of -28 cmH_2O were performed. This resulted in an exponential increase in pO_2 and V_T in the beginning of the treatment interval but resulted in no significant long-term improvement in respiratory parameters after the end of the administration time (Figure 24).

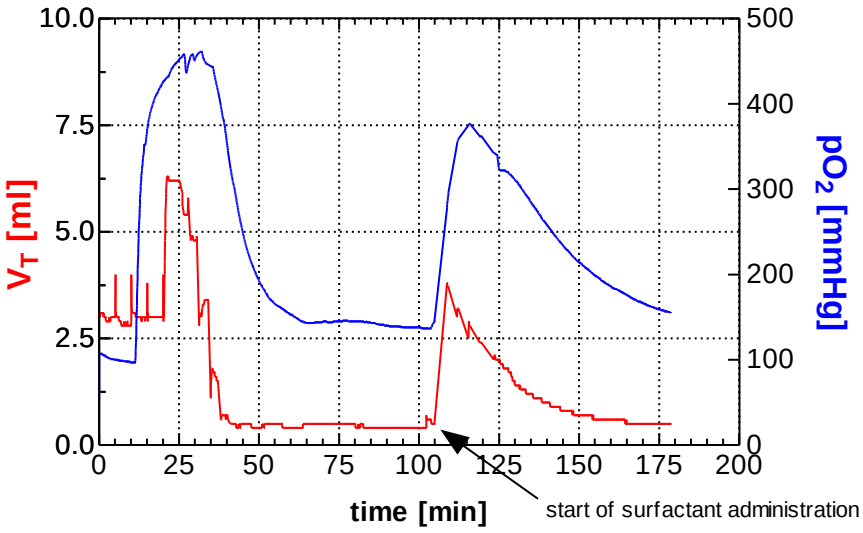

Figure 24: V_T and pO_2 in lavaged IPLs with decreased EIP and DI. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), EIP was set to -20 cmH_2O. 28 DI were performed during surfactant administration time, n = 1.

In another approach, the respiratory rate was lowered from 80 to 30 breath/min before the first bronchoalveolar lavage was performed. This led to a similar decline in V_T and pO_2 during and after BAL performances as with a RR setting of 80 breath/min. After an exponential increase in the beginning of the aerosol administration, the respiratory parameters were impaired immediately. No significant improvement in the respiratory parameters after surfactant treatment was noticed (Figure 25).

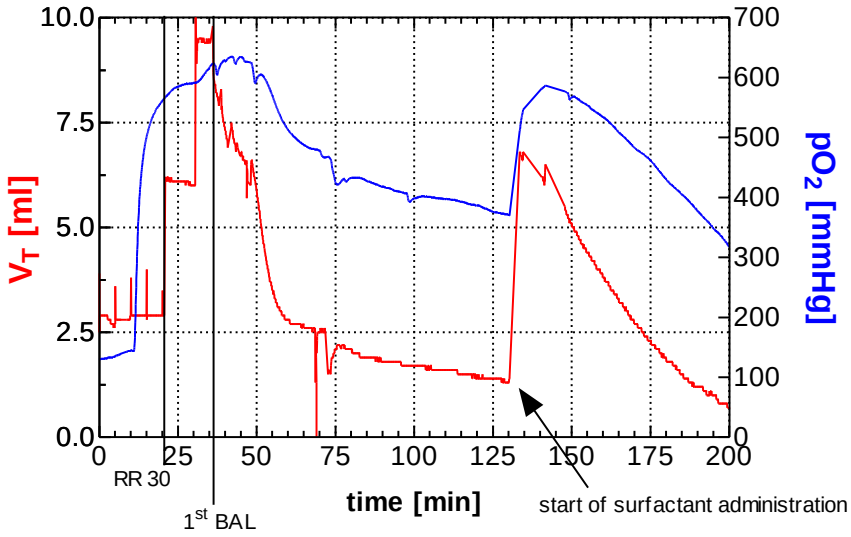

Figure 25: V_T and pO_2 in lavaged IPLs with reduced RR. Repetitive BALs were performed every 5 min after RR was set to 30 breaths/min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered, n = 1.

Surfactant administration was also assessed macroscopically. It was noticed that the distribution of surfactant in the lung lobes was non-uniform. Within the first period of aerosol administration, exogenous surfactant was primarily deposited in the pars cranialis of the pulmo sinister (Figure 26 a)) and in the lobus cranialis pulmonis dextri and lobus medius pulmonis dextri (Figure 26 c)). The elasticity of the left lung was generally restored faster than a recovery of the breathing motion was seen in the right lung. Nearly complete plump lung tissue was seen in every lung lobe towards the end of exogenous surfactant administration (Figure 26 b) and d)).

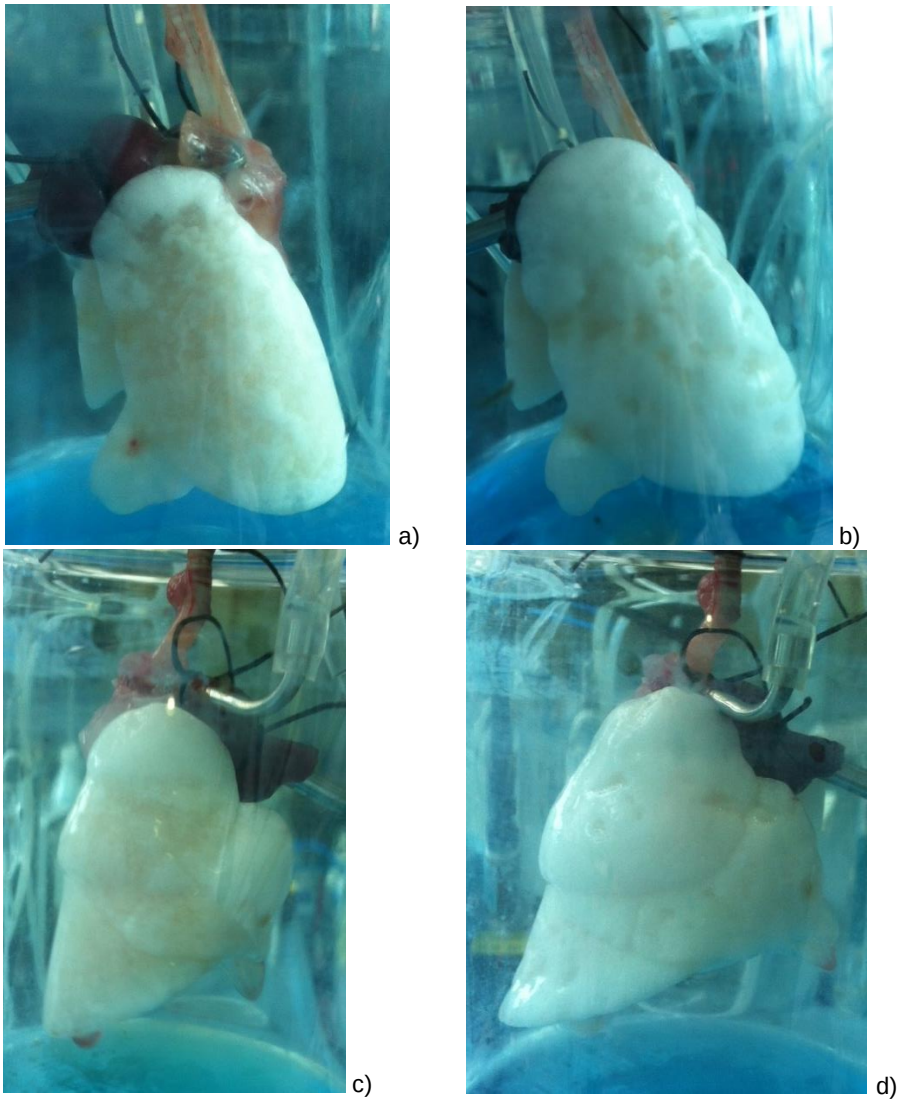

Figure 26: Lavaged IPLs during and after rSP-C administration. Lungs were repeatedly lavaged before a 1 h resting period of impaired lung function was conducted. Surfactant administration initially led to an opening of the upper parts of both lungs. a) Opening of pulmo sinister (pars cranialis), b) Restoration of the left lung after rSP-administration, c) Opening of lobus cranialis pulmonis dextri and lobus medius pulmonis dextri, d) Restoration of the right lung after rSP-admininstration,

For an estimation of the administered surfactant dose, filter samples were taken with a vacuum flow rate of 0.22 ml, in order to emulate the V_T of a lavaged lung. The mean deposited surfactant mass per puff amounted to 0.022 mg (n=3, SD=0.005). With regard to the entire administration phase of 250 puffs of aerosol across IPL trials, a mean of 5.5 mg surfactant was deposited on the filter. With regard to the

mean test animal body weight of 272 g, a concentration of 20 mg/kg bw was assumed that was administered to the lungs.

4.3.1. Oxygenation

Control lungs showed a slight increase in pO_2 levels 60 min after exogenous surfactant administration which was not significant compared to the same controls before the start of surfactant administration (Figure 28).

Administration of surfactant aerosols had a significant effect on pO_2 levels. Protein-free placebo surfactant, showed a significant improvement 10 min after surfactant administration. Furthermore, 60 min after placebo (no protein content) administration, the increase in arterial pO_2 remained significant. Prior to BAL performance, the oxygen level after placebo administration was at 51% compared to baseline values at 10 min following administration, and 54% after 60 min, respectively (Table 8).

Administration of 20% surfactant resulted in a significant increase in pO_2 after 10 and 60 min (Figure 28). This increase was almost fourfold compared to untreated controls after 10 min and dropped to a factor of 2.6 at 60 min after surfactant administration (Table 8). When oxygenation levels after 20% surfactant administration were compared to baseline values, a recovery of 76.9% after 10 min and 75.7% after 60 min was found (Table 8). Administration of 20% surfactant did not result in a significantly higher increase in pO_2 compared to administration of placebo surfactant (Figure 28).

The highest increase in pO_2 compared to untreated controls was a 4.7-fold increase 10 min after administration of 100% surfactant. Even at 60 min after administration, 100% surfactant resulted in a 3.6-fold increase in pO_2 levels (Table 8). When pO_2 levels after 100% surfactant administration were compared to other surfactant formulations tested, significant differences were detected. The placebo surfactant was significantly less effective in pO_2 increase at all times after surfactant

administration. A significantly greater effect of 100% surfactant compared to the 20% surfactant formulation was only seen 60 min after administration (Figure 28). The administration of 100% surfactant resulted in a recovery of baseline values of 88.8% at 10 min, and 103.5% at 60 min after the end of administration, respectively (Table 8). A significant increase in pO_2 of 14.6% after administration of 100% surfactant between 10 and 60 min was noticed (Figure 28). After administration of placebo and 20% surfactant, almost no further effects on pO_2 levels was seen between 10 and 60 min after the completion of dry powder administration, showing increases of 2.7% and 1.2%, respectively.

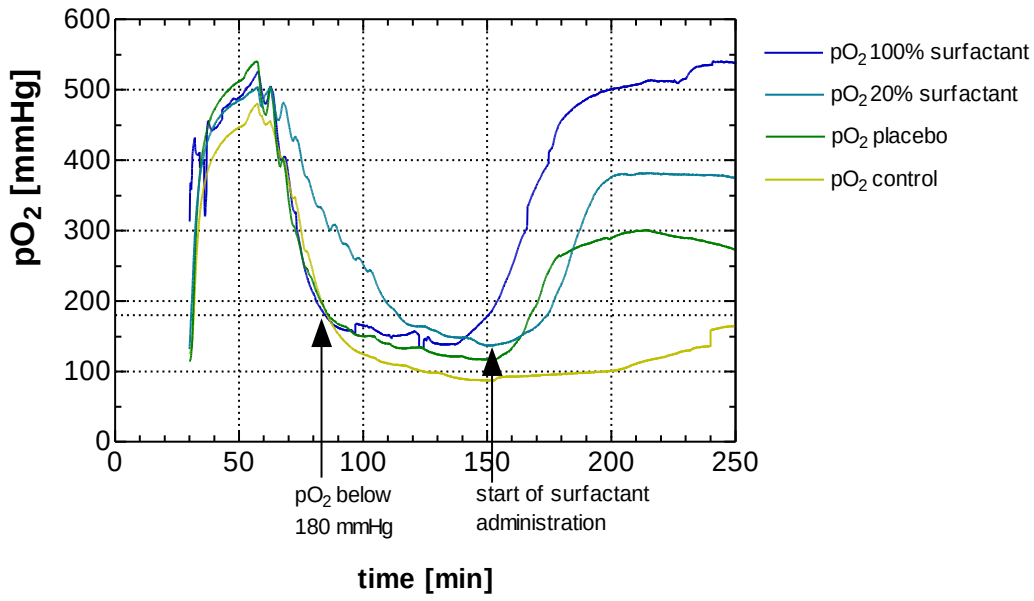

Figure 27: PO_2 during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered, n = 4 for every group.

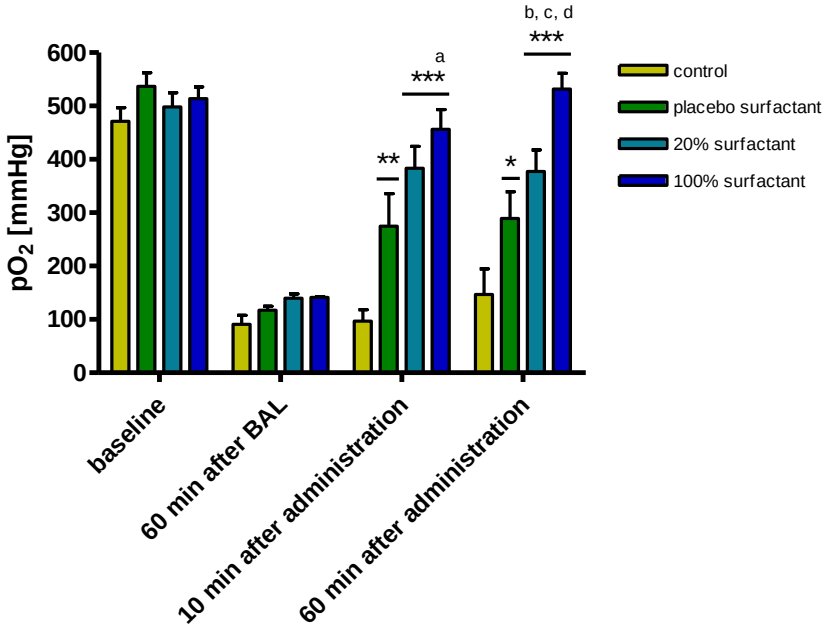

Figure 28: PO_2 after BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by two-way repeated measures ANOVA. Values are given as means + SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control, [a] $p < 0.01$ and [b] $p < 0.001$ vs. placebo, [c] $p < 0.01$ vs. 20% surfactant, [d] $p < 0.05$ vs. 100% surfactant 10 min after administration, n = 4 for every group.

Table 8: Percentage of increase in pO_2 after surfactant administration. Total and relative pO_2 values in comparison to untreated controls. Surfactant was administered to ventilated IPLs 1 h after the lung function was impaired by BALs, n = 4 for every group.

	control	placebo	20% surfactant	100% surfactant
baseline	471.00	536.50	498.00	513.75
SEM	25.93	25.88	26.57	21.75
60 min after BAL	90.75	117.25	139.50	141.00
SEM	16.77	7.43	8.39	1.87
related to baseline [%]	19.27	21.85	28.01	27.45
10 min after administration	96.75	274.50	383.00	456.25
SEM	21.74	61.49	41.14	37.05
related to baseline [%]	20.54	51.16	76.91	88.81
related to control [%]	100	283.72	395.87	471.58
60 min after administration	146.75	289.00	377.00	531.50
SEM	48.16	50.42	40.83	29.89
related to baseline [%]	31.16	53.87	75.70	103.45
related to control [%]	100	196.93	256.90	362.18

4.3.2. Tidal Volume

Administration of surfactant led to a direct increase in V_T. As Figure 29 shows, the increase in V_T occurred immediately after administration and did not increase any further until the end of IPL trials. Controls showed a slight increase in V_T 60 min after administration compared to 10 min after administration, which was not significant (Figure 30).

A clear though non-significant increase in V_T was seen after placebo surfactant administration. This increase resulted in a V_T four times that found in untreated controls (0.85 ml after placebo treatment vs. 0.2 ml in controls) right after surfactant administration and in a decrease to a mean V_T of 0.63 ml (decrease of 26%) at the end of trials.

Administration of surfactant formulation with 20% rSP-C did not result in a significant enhancement in V_T compared to untreated controls. V_T increased to 1.33 ml 10 min after treatment and was measured to be 1.15 ml at 60 min after 10% surfactant administration, respectively.

After 100% surfactant administration, V_T was 2.10 ml (±1.46), and 2.08 ml (±1.39) 60 min after administration, respectively. This enhancement was also significant compared to the administration of placebo surfactant at all times.

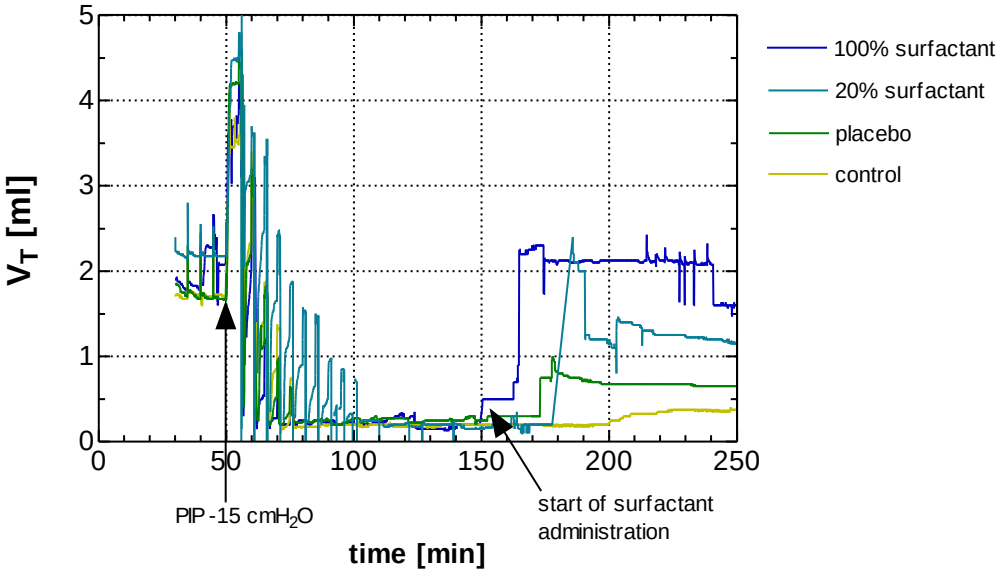

Figure 29: V_T during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered, n = 4 for every group.

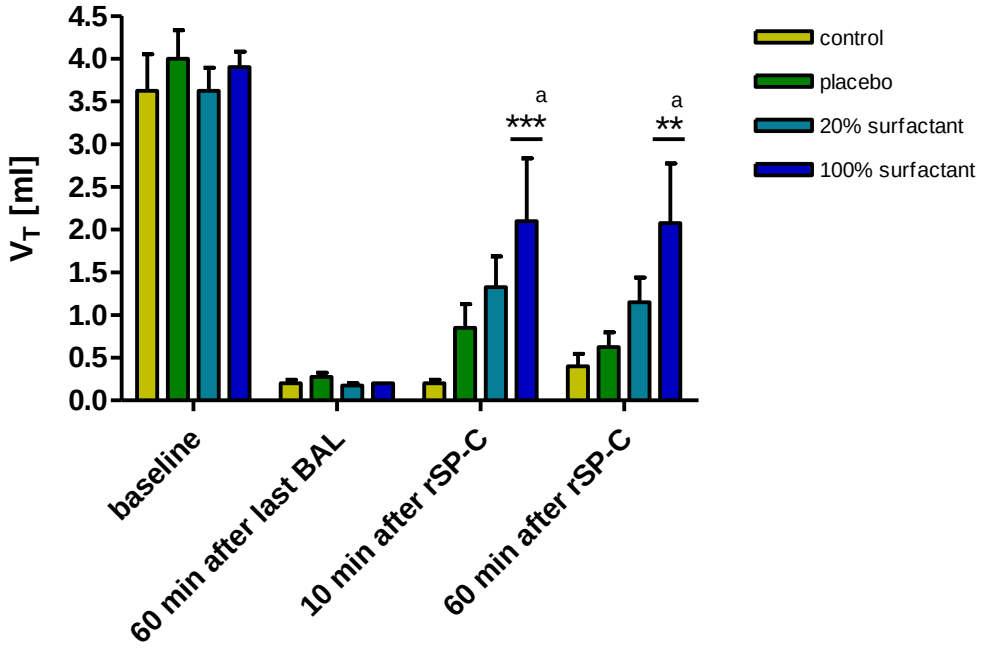

Figure 30: V_T during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by two-way repeated measures ANOVA. Values are given as means + SEM, ** $p < 0.01$, *** $p < 0.001$ compared to controls, [a] $p < 0.05$ vs. placebo, n = 4 for every group.

4.3.3. Dynamic Lung Compliance

Dynamic lung compliance was significantly improved after administration of 100% rSP-C in comparison to untreated controls. Exogenous surfactant administration led to no significant improvement when compared to administration of protein-free surfactant or the surfactant preparation with reduced protein content (Figure 31).

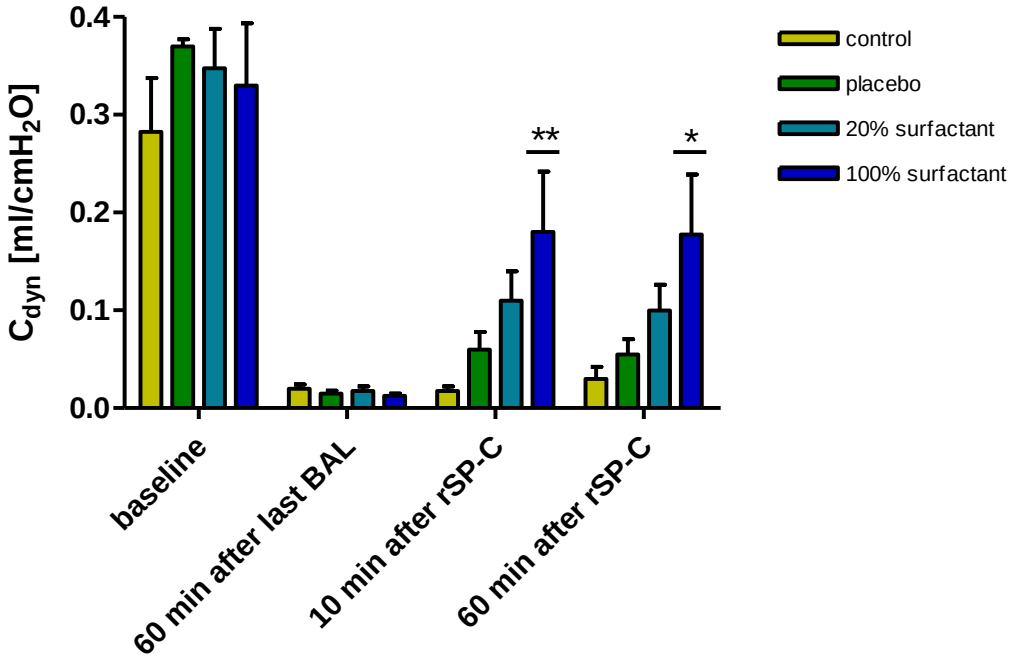

Figure 31: C_{dyn} during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by two-way repeated measures ANOVA. Values are given as means + SEM, n = 4 for every group. * p < 0.5 ** p < 0.01, compared to controls.

4.3.4. Lung Resistance

The administration of exogenous surfactant did not lead to altering of lung resistance (R_L) at any point. A significant reduction in R_L compared to the control group could be found in the group of lungs that received 100% surfactant thereupon. This effect was only seen in one group 1 h after the end of bronchoalveolar lavages.

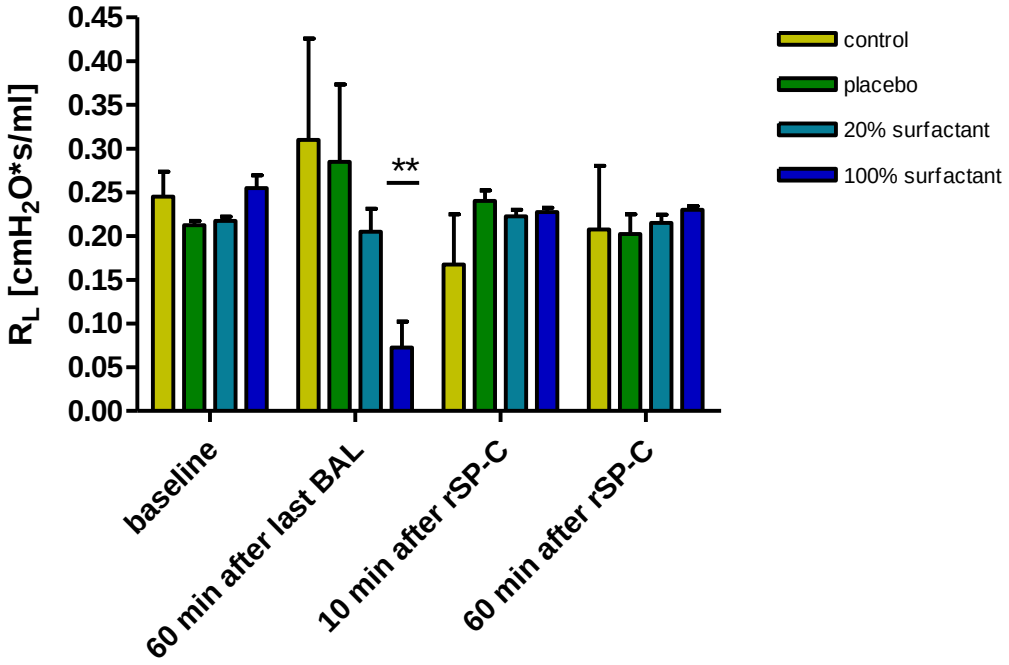

Figure 32: R_L during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by two-way repeated measures ANOVA. Values are given as means + SEM, n = 4 for every group. ** p < 0.01, compared to controls.

4.3.5. Lung Weight

No significant differences in weight could be found between groups at any time (Figure 33). In contrast to significant weight changes after BAL performances, surfactant administration did not result in weight gain (Figure 33).

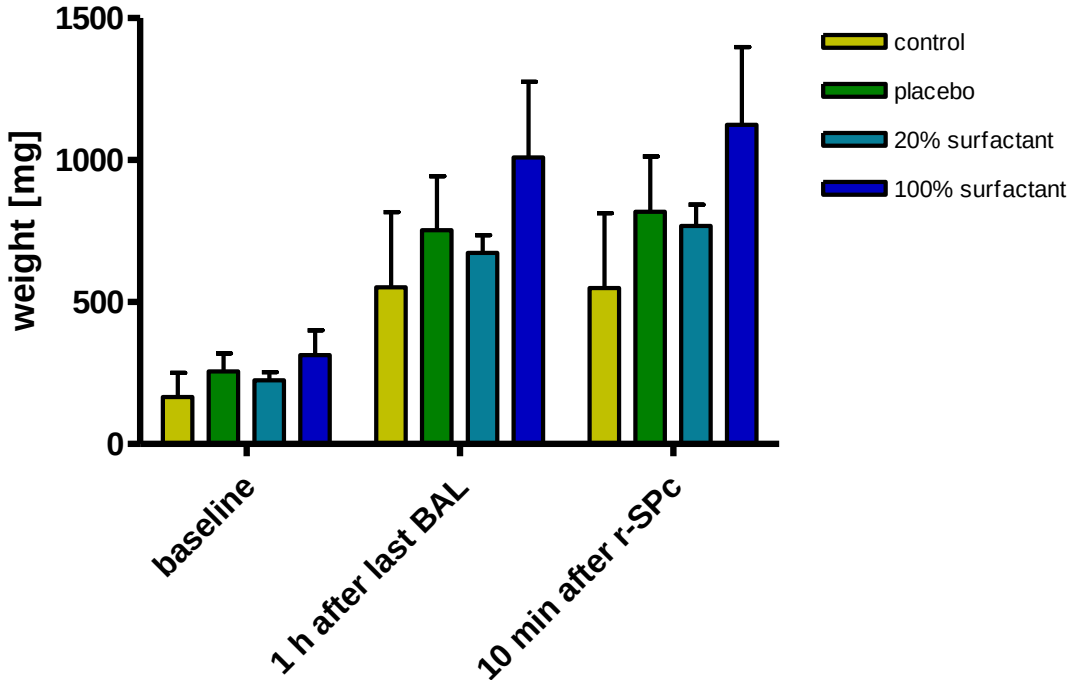

Figure 33: Lung weight during BALs and surfactant administration. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by two-way repeated measures ANOVA. Values are given as means + SEM, n = 4 for every group.

4.3.6. Oxygenation in IPLs and the RLL model

The oxygenation levels of IPL trials and RLL trials were compared to evaluate the correlation of both models. Before BAL performances pO_2 levels of 513 mmHg in IPL and 511 mmHg in RLL trials were reached (Figure 34).

On an average, 6 BALs were performed in IPL trials to reach pO_2 levels ≤ 180 mmHg, while only 4 to 5.75 BALs were needed to achieve the same decrease in RLL trials.

IPLs showed an average pO_2 of 456.3 mmHg 10 min after surfactant administration and a pO_2 of 531.5 mmHg 60 min after treatment, respectively. This resulted in a 3.2 fold post-treatment increase in pO_2 (pre-treatment pO_2 155 mmHg, post-treatment pO_2 493.9 mmHg). In RLL trials, a mean pO_2 of 447.4 mmHg was reached 10 min

after surfactant administration and at 60 min after treatment pO_2 increased to 473.3 mmHg, resulting in a 7.4 fold post-treatment increase in pO_2 (pre-treatment pO_2 61.5 mmHg, post-treatment pO_2 456.9 mmHg).

In the comparative RLL study, 1 animal died during BAL performances. Another animal deceased before surfactant administration. One animal died right after surfactant administration while additional 6 test animals did not reach the end of the study. All in all, 7 out of 16 animals reached the end of the study. From these, the data of 4 animals were chosen to compare SEMs in IPL and RLL trials (Figure 34). The data show that SEMs in IPL trials are greater than in RLL trials when the same statistical population is provided.

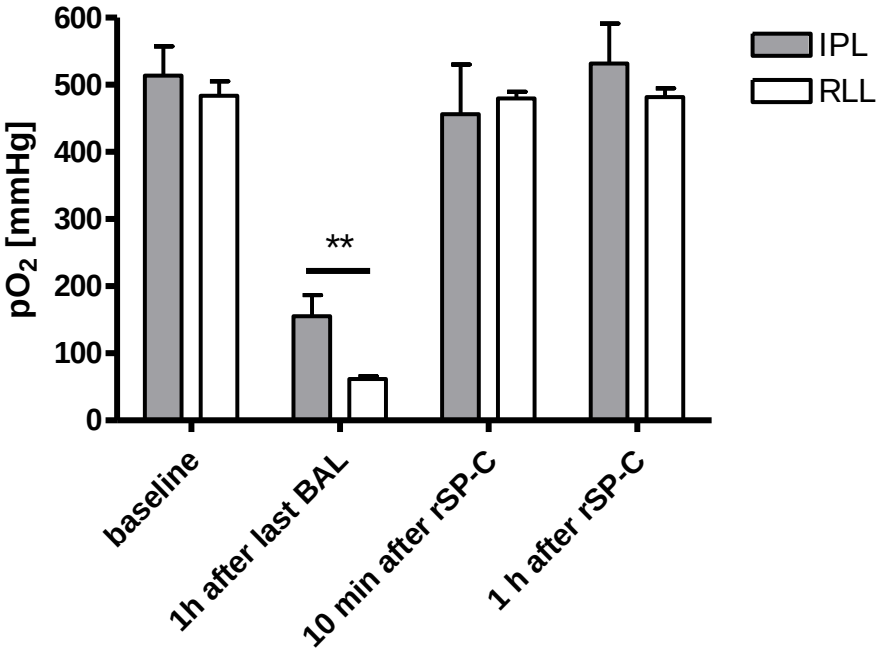

Figure 34: PO_2 levels in RLL and IPL trials. Repetitive BALs were performed every 5 min. After 1 h of impaired breathing function (pO_2 < 180 mmHg), aerosolized surfactant was administered. Data were analyzed by Student's t-test. Values are given as means + SEM, n = 4 for every group in IPL trials. In RLL trials at baseline (n = 15), 1 h after last BAL (n = 13), 10 min after rSP-C administration (n = 12), 1 h after rSP-C administration (n = 7).

5. Discussion

5.1. Ventilation of IPLs as Secondary Surfactant Deficiency Model

Ventilation settings are very important components in RDS and ARDS treatment. Unlike any other treatment, mechanical ventilation can have adverse effects. On the one hand, very low-volume ventilation can lead to atelectrauma. On the other hand, very high-volume ventilation can lead to volutrauma due to overstretching of parts of the lung tissue (ALBAICETA and BLANCH, 2011). In this context, the use of high positive end-expiratory pressures in rats seems to be a very effective strategy in preventing ventilator-induced lung injuries (VALENZA et al., 2003).

An increase of positive inspiratory pressure during mechanical ventilation in animal models for ARDS is needed to overcome pulmonary rigidity (MENK et al., 2015). Typical mechanical ventilation of IPLs is performed with an end-inspiratory to end-expiratory pressure ratio of -8.8/1.8 cmH_2O with which lungs are viable for at least 180 min (UHLIG and TAYLOR, 1998; UHLIG and WOLLIN, 1994). Our trials showed that IPLs are also viable for at least 210 min with an EIP/EEP ratio of -15/-3.6 cmH_2O. End-inspiratory pressure was set to -15 cmH_2O right before the start of bronchoalveolar lavage performances as it is thought that this setting helps to prevent total alveolar collapse after partial wash out of endogenous surfactant. Moreover, an EIP of -15 cmH_2O did not lead to edema formation in our study which is a serious risk in mechanical high volume ventilation (FU et al., 1992).

It is sensible to set the end-expiratory pressure in IPL trials to a low level, as it is known from positive ventilation trials that a high positive end-expiratory pressure setting prevents alveolar collapse at the end of expiration (HALTER et al., 2003). Therefore, the lowest feasible EEP setting of -3.6 cmH_2O was chosen in IPLs for surfactant deficiency modeling.

Mechanical ventilation itself can be associated with pulmonary edema formation. Relative lung weight as a result of different EIP settings was assessed in the IPLs. Moderate linear weight gain in IPLs is common as artificial perfusion of lung tissue leads to higher vascular permeability and slight edema formation over time. The onset of edema formation is dependent on the dimension of end-inspiratory pressure. With an EEP of -3.6 cmH_2O, an EIP of -17 cmH_2O only resulted in slow edema formation, whereas a lower end-inspiratory pressure of -26 cmH_2O resulted in immediate edema formation within 2 min of ventilation (see Figure 7). Edema formation is highly undesirable in surfactant efficacy testing as plasma and blood proteins inhibit surfactant function (NOTTER, 2000). In blood-free IPLs, artificial buffer is enriched with bovine serum-derived albumin that infiltrates the lung tissue during edema development. In bubble surfactometer experiments, 0.4 or 0.75 mg/ml of lung surfactant was mixed with 5 or 10 mg/ml albumin, respectively (HOLM and NOTTER, 1987). At both surfactant concentrations, the minimum surface tension was immediately increased from 20 to 45 mN/m, with the higher albumin concentration not causing any further aggravation (HOLM and NOTTER, 1987). These results indicate that the size of edema formation does not correlate with the degree of surfactant impairment.

In a study investigating the impact of high tidal volumes on lung edema clearance, IPLs were ventilated with inspiratory pressures of 8, 20, or 35 cmH_2O (LECUONA et al., 1999). Lung edema clearance was impaired after ventilation with the high inspiratory pressure. It was assumed that the ability to clear edema was impaired by high inspiratory pressures due to inhibition of active Na+ transport in the alveolar epithelium (LECUONA et al., 1999). Our experiments showed that a decrease in V_T could only be noticed much later after marked increases in lung weight and concomitant edema formation. This does indicate that IPLs are able to maintain pulmonary functions after the onset of edema formation. A linear increase in lung weight represents an increase in vascular leakage and might be associated with the formation of interstitial lung edema if lung clearance is insufficient. In contrast, complete lung edema formation resulted in a delayed exponential decrease in tidal volume which means that breathing ability after complete lung edema formation is

completely impaired (see Figure 6). Complete lung edema formation led to fluid accumulation in the trachea from a macroscopic perspective as well, whereas interstitial edema formation could not be assessed macroscopically.

The baseline values of moderate high pressure ventilated IPLs were compared to respiratory parameters of IPLs ventilated under normal conditions. With an end-inspiratory to end-expiratory pressure setting of -15/-3.6 cmH_2O in our trials, the mean tidal volume was 3.79 ml, dynamic lung compliance amounted to 0.38 ml/cmH_2O, and lung resistance was 0.23 cmH_2O*min/ml. In comparison, when IPLs were ventilated with an EIP/EEP ratio of -6/-2 cmH_2O, V_T amounted to 2 ml, while C_{dyn} was measured to be 0.5 ml/cmH_2O, and R_L was 0.2 cmH_2O*min/ml (UHLIG and TAYLOR, 1998). These data show that even doubling the respiratory volume under negative pressure ventilation only correlates with a decline in dynamic lung compliance of 24%. Not surprisingly, lung resistance was not affected by the increase in respiratory volume. This indicates that the lung function can be maintained with our ventilation setting.

Deep inspiratory breaths with an EIP/EEP ratio of -20/-3.6 cmH_2O were executed between BALs to recruit remaining surfactant from the alveoli. DI breaths led to a raise in tidal volume. These recruitment maneuvers are widely known from animal models for investigation of RDS or ARDS (HALTER et al., 2003). Recruitment maneuvers with increased inspiratory pressure open collapsed alveoli and are also applied in the clinical treatment of acute respiratory distress syndrome and respiratory distress syndrome to support oxygenation in a so called “open lung” approach (CHIUMELLO et al., 2015; HALTER et al., 2003; SCHREITER et al., 2016). The recruitment maneuvers performed in the IPL trials showed to be effective in partial restoration of lung function which was seen in an immediate increase in dynamic lung compliance after deep inspiration breath performances (see Figure 12).

5.2. Respiratory Parameters in Lavaged IPLs

Warm saline is commonly used for bronchoalveolar lavages to provoke secondary surfactant deficiency in animal models (HAFNER and GERMANN, 1999; MATUTE-BELLO et al., 2008; MENK et al., 2015). In the study by Häfner and Germann, the influence of the lavage volume on declines in pO_2 was examined. Male Sprague-Dawley rats were positively pressure ventilated with a PIP/PEEP ratio of 15/2 cmH_2O. It was found that a scheme of 7 lavages with 8 ml of warm saline was most effective in decreasing pO_2 (HAFNER and GERMANN, 1999). These findings are in accordance with the results of the present *ex vivo* study (IPLs) in which a mean of 6 (± 2.33) x 8 ml warm saline bronchoalveolar lavages was needed for the oxygen level to drop to a state of moderate ARDS.

A single BAL resulted in an average tidal volume of 2.8 ml with which IPLs were perfectly capable of breathing (see Table 4). Compared to standard V_T values for IPLs which range from 1.8 to 2 ml when ventilated with an end-inspiratory pressure to end-expiratory pressure ratio of -6 to -8.81/-1.83 to -2 cmH_2O and 80 breaths/min (HOFMANN et al., 2006; UHLIG and TAYLOR, 1998; UHLIG and WOLLIN, 1994), IPLs in our trials showed an above-average respiratory capability after the first lavage when ventilated with a EIP/EEP of -15/-3.6 cmH_2O and 80 breaths/min. This might be the reason why only a second BAL resulted in a significant decrease in pO_2 (Figure 10). A decrease of dynamic lung compliance over time in IPL trials is physiologically based as the lung tissue tightens given a lack of deep inspiration breaths. The performance of recruitment maneuvers with a single deep inspiration breath every 5 min proved to be effective as C_{dyn} improved immediately thereafter. In contrast to the progression of pO_2 decrease, the first BAL resulted in a significant drop of tidal volume and dynamic compliance. Any additional bronchoalveolar lavage resulted in a further decrease in V_T and C_{dyn} which was then followed by a reduction in pO_2. As the 1st and 2nd BAL resulted in a mean V_T of 1.8 ml and did not lead to any increase of lung resistance, it can be assumed that lavage performance was sufficiently gentle and thus did not result in collapsed bronchial tubes.

The significant increase in lung resistance after the performance of a 3rd BAL as well as the slight increase after performance of a 4th, 5th and 6th lavage may result from a short period of increased humidity in the trachea rather than from pathophysiological changes. This is very likely as the process of lung resistance did not consistently show a high level (see Figure 14). Also, following a significant increase in R_L after the 3rd BAL, lung resistance sank after performance of the subsequent lavage (see Figure 15). This high degree of moisture in the upper airways resulted from BAL performance. Shortly after the completion of lavages, R_L descended to a normal value (see Figure 15). Strikingly, the resistance level was found to be below baseline level one hour after the last bronchoalveolar lavage performance. Even if this impact on resistance to airflow was not significant, this effect may have resulted from the extension of small airways by saline lavage.

When V_T was equal or below 0.4 ml, no more BALs were performed and deep inspiratory breaths were switched off to prevent additional surfactant recruitment. In general, C_{dyn} did not increase after the end of bronchoalveolar lavages. In some cases however, a slight improvement of dynamic lung compliance occurred prior to surfactant administration and was followed by an increase in pO_2. In these cases an extra warm saline lavage was performed.

When post lavage data were compared to baseline values, a clear significant decrease in C_{dyn} of 96.7% could be seen (see Table 5). This tremendous change stands in clear opposition to the comparatively small change in lung resistance of 6.2% (Table 6). These findings indicate that bronchoalveolar lavages *ex vivo* effectively affect the alveolar parenchyma rather than causing any adverse impact on the upper airways.

The amount of relative weight gain is equal to the amount of remaining saline in the lung after BAL performance. The first lavage led to a significant mean increase in lung weight, whereas successive BALs only caused very slight weight gain (see Figure 16). While handling the lavages, it was furthermore observed that performing the first bronchoalveolar lavage is more difficult than the following ones. This finding is in agreement with RLL trials and suggests that the lung integrity is mostly affected

by the performance of a single BAL. This assumption is also substantiated by the significant abrupt decrease in C_{dyn} after the performance of the first lavage (see Figure 13). Immediate impairment of lung elasticity after first BAL performances was also found in another study in which pulmonary compliance was assessed (MENK et al., 2015). The extent of lowering in dynamic lung compliance also gives information about the efficacy of BAL performances. Thus, it can be stated that bronchoalveolar lavage performances caused effective leaching of surfactant from the alveolar parenchyma in IPLs as C_{dyn} decreased significantly during repeated lung lavage. Performance of the established bronchoalveolar lavage scheme in IPLs did not lead to edema formation at any time which would have involved an exponential increase in lung weight, inter alia (see Figure 16).

5.2.1. Histologic Analysis of Lavaged IPLs

The histological examination largely showed normally structured, viable lung tissue. This indicates that repetitive warm saline BALs were performed gently and did not harm the lung tissue structure. Moreover, the ventilation setting with a relatively low end-inspiratory pressure of -15 cmH_2O did not damage the integrity of the alveoli within the trial duration of 240 min. Dead cells could only be found scattered throughout the intact tissue structure (see Figure 17). Extensions of alveolar spaces were observed (see Figure 18). These resulted from artificial perfusion of lung tissue and are known from IPL trials. IPLs were perfused with artificial buffer which is not stained in HE stains. Consequently, no assessment of pulmonary edema formation could be executed. The morphologic differences between compressed and extended intercellular spaces are mutually dependent. Over the course of the repetitive BAL performances, surface tension on alveoli increased which was visible in decreased C_{dyn}. As a result of this, contracted alveolar spaces caused other alveoli to stretch, in turn causing an extension of neighboring alveolar spaces (see Figure 18).

5.2.2. Biochemical Analysis of Lavage Fluids

One way of assessing the cytotoxicity of repeated warm saline lavages is the determination of lactat dehydrogenase in the perfusate of IPLs. Using this technique, lungs are considered physiologically intact if the amount of LDH in the perfusate flowing into the lung tissue is equal to the amount of LDH in the perfusate flowing out of the lung tissue (HOFMANN et al., 2006). Another technique was used by Barr et al. They analyzed lactat dehydrogenase in bronchoalveolar lavage fluids of rat-IPLs to check the integrity of the epithelium after treatment with [^{3}H]-choline and a secretagogues (BARR et al., 1988). IPLs were ventilated with positive pressure at 60 breaths/min with 5% CO_2 / 95% O_2, a tidal volume of 2.5 ml and an end-expiratory pressure of 2 cmH_2O, while the perfusion rate was 10 ml/min (BARR et al., 1988). No difference in LDH was observed between treated lungs and controls. This showed that epithelium integrity was affected neither by the treatment nor by the lavage technique, in which three lavages were performed consecutively with 0.15 M saline (40 ml/kg bw) at 22°C and each volume was instilled and withdrawn three times (BARR et al., 1988). These results are in accordance with the results obtained from our IPL trials, which showed no increase in LDH release in bronchoalveolar lavage fluid after repeated lavages (see Figure 20).

However, a significant difference in cytotoxicity could be observed after the performance of a fifth BAL between RLL and IPL trials (see Figure 20). The noticeably higher cytotoxicity in the fifth BALF of RLL trials may result from at least two major differences in the model's setup. One reason could be the integration of an extra time interval of 5 min the RLL lavage compared to the IPL lavage scheme prior to the performance of the fifth lavage. Usually, four BALs are performed in RLL trials at a time interval of 5 min. After the fourth bronchoalveolar lavage, blood is sampled for determination of blood gases. This lavaged lung status is then maintained for 10 min after which another blood sample is taken to identify the drop in pO_2. This prolonged time frame in RLL trials before performing optional BALs is needed to save blood for sampling and also for detection of a decrease in oxygen. However, the

prolonged time interval might also have an impact on epithelial cytotoxicity as the lung tissue collapses and compresses. It can also be assumed that positive pressure ventilation in RLL trials is more harmful to the lung tissue than negative pressure ventilation in IPL trials. It is known that negative pressure ventilation induces less edema formation compared to positive pressure ventilation (DRITSAS et al., 1969; SKABURSKIS et al., 1989; UHLIG and TAYLOR, 1998). Thus, it is conceivable that after the fourth lavage in a short period of time, the lung tissue in RLL trials is more irritated because of positive pressure ventilation with a PIP/PEEP ratio of 26/6 cmH_2O than it is in IPL trials where it is ventilated with a negative EIP/EEP of 15/3.6 cmH_2O. Furthermore, the significant increase in LDH at the end of RLL trials corroborates the assumption that positive pressure ventilation might have a greater impact on the lung's epithelial structure than negative ventilation (see Figure 20).

The analysis of protein released from damaged epithelial lung cells revealed no increase between successive bronchoalveolar lavage fluids. The amount of protein content was highest in the first lavage fluid compared to the four following BALFs (Figure 21). Menk et al. performed repeated pulmonary lavage to reproduce histological and physiological findings of acute lung injury (MENK et al., 2015). In their study, male Sprague-Dawley rats (300 - 350 g) received pressure controlled positive ventilation (PIP/PEEP: 14/2 cmH_2O, 30 breaths/min, inspiratory/expiratory ratio 1:2) and five to six warm saline BALs (37°C, 30 ml/kg bw) (MENK et al., 2015). After pO_2 was < 150 mmHg, a recruitment maneuver was performed (PIP: 34 cmH_2O) after which ventilator settings remained unchanged (PIP/PEEP: 26/6 cmH_2O) for 240 min (MENK et al., 2015). Protein concentration was measured in the first and the final bronchoalveolar lavage fluid using the bicinchoninic acid assay (MENK et al., 2015). The protein concentration in the first lavage sample was significantly increased (ca. 0.8 mg/ml) compared to pure saline (n.d.) (MENK et al., 2015). This result is fully in line with our finding of increased protein content in the first BALF (3600 mg/ml). This might indicate that mechanical ventilation is stressful to the lung tissue. The comparison shows that protein content in the first bronchoalveolar lavage fluid of IPLs is much higher than in *in vivo* trials. One reason for this might be that extraction of the heart-lung block and *ex vivo* perfusion in

combination with a switch from positive to negative ventilation causes substantial extra stress to the lung tissue. As a result, large amounts of dead cells and released proteins are washed out of the organ with the first BAL. Another reason for the 4500-fold higher protein concentration in lavage fluids of IPLs may be an increase in the transfer of albumin from the artificial buffer via the alveolar-capillary barrier into the lung tissue.

Performances of saline lavages cause a reduction of surfactant lining in alveoli. This leads to reduced breathing ability and might also result in atelectatic lung tissue as the alveoli lose their natural constitution (DUGGAN and KAVANAGH, 2005). It is furthermore conceivable that atelectatic lung tissue leads to stenosis of blood vessels. These pathologic changes may promote an increase in vascular permeability. In IPL trials, this enhanced permeability results in an increase of protein in BALF. Teng et al. reported an increase in vascular permeability in IPLs. They perfused IPLs with standard artificial Krebs-Henseleit-solution containing 4% of bovine serum albumin. Left lung perfusion was subjected to 60 min of ischemia and 30 min of reperfusion. After at least 150 min, at the end of trials, three lavages were performed in each lung separately, using 2.5 ml of physiological saline for each bronchoalveolar lavage. A total of 12 ml of lavage fluid was recovered. A significant increase in albumin in BALF was found in IPLs after ischemia induction (TENG et al., 2004).

Furthermore, Menk et al. stated that increasing numbers of BALs led to a significant increase in protein content in bronchoalveolar lavage fluids because of heightened permeability of the alveolar-capillary membrane (MENK et al., 2015). This stands in strong contrast to the invariant protein contents found in repetitive BALFs of IPLs and could have several causes. The increase in permeability of the alveolar-capillary barrier is very much dependent on the performance of bronchoalveolar lavages and also on ventilation settings. Moreover, our experiments showed a 71% decrease in C_{dyn} after the first lavage and 96% decrease after performance of the last BAL (see Table 5). Menk et al. only analyzed C_{stat} at the end of a trial which does not explain the exact time point of C_{dyn} impairment. It is reasonable to think that C_{dyn} behaves

differently under pressure-controlled positive ventilation than under negative pressure conditions and that C_{dyn} is only impaired at a later stage. Considering this further, large amounts of albumin from the artificial buffer are analyzed in BALFs from IPL trials and contribute to the high amount of protein content in BALFs (see Figure 21). Alveolar-capillary membrane injuries are usually involved in the pathophysiological changes brought about by ARDS. These vascular dysfunctions are associated with the induction of pulmonary edema formation and the release of blood proteins (WILLSON and NOTTER, 2011). In this context, an increase in protein in BALF at the end of IPL trials is in accordance with the slight relative weight gain towards the end of IPL trials (see Figure 21 and Figure 33).

It can be assumed that negative or positive ventilation settings in IPL and RLL trials did not impact acute inflammation settings in the secondary surfactant deficiency models IPL and RLL. With regard to BAL performances in both models, it appears that BALs were performed equally gently, as TNF-α was not detectable in the single bronchoalveolar lavages. Although, the TNF-α concentration at the end of IPL trials seems extremely significant compared to the final lavage fluid obtained from RLL trials, t-test results showed a p-value of only 0.0423 (Figure 22). This low significance level resulted from extreme variations in TNF-α-levels in IPL trials. Based on the relatively low levels of TNF-α in the pg/ml range, only low acute inflammation levels might have been induced by repetitive BAL performances in IPLs. Compared to a study by Pilla et al., TNF-α in bronchoalveolar lavage fluids was about 50 times higher than in our IPL trials. In this study, TNF-α was measured in a rat model of brain death (PILLA et al., 2013). A saline BAL was performed 5 min after brain death was determined. These striking differences in TNF-α-levels compared to our *ex vivo* study might have resulted from great differences in the experimental setup for cytokine measurements. Pilla et al. determined TNF-α immediately after the decease of the test animals, while cytokine measurements in IPL trials were performed in viable lungs. Interestingly, the standard deviation of TNF-α in BALF in the trials from Pilla et al. was also immense and accounted for about 50% of the variation (PILLA et al., 2013).

In another study, transcriptional induction of TNF-α was analyzed in the lung tissue of mechanically ventilated rats (MENK et al., 2015). In that study, male Sprague-Dawley rats were ventilated with a PIP/PEEP of 14/2 cmH_2O. Respiratory failure was induced by performance of five to six saline BALs (37°C; 30 ml/kg) until pO_2 was below 150 mmHg (MENK et al., 2015). After the end of lavage performances, a recruitment maneuver was conducted by raising PIP to 34 cmH_2O for three breathing-cycles (MENK et al., 2015). Then, PIP/PEEP was set to 26/6 cmH_2O until the termination of the study at 240 min (MENK et al., 2015). The expression of messenger ribonucleic acid (mRNA) levels of TNF-α was measured in total RNA of homogenized lung tissue by quantitative real-time polymerase chain reaction (MENK et al., 2015). Menk et al. stated that repetitive BALs led to a significant induction of TNF-α mRNA after 240 min of mechanical ventilation (MENK et al., 2015). In contrast to cytokine mRNA induction in BALF, no inflammatory cytokines were found in blood circulation (MENK et al., 2015). These findings are in contrast to the lack of TNF-α induction in our *in vivo* trials (see Figure 22) and could result from different ventilation settings. It can be assumed that the maintenance of a stable PIP/PEEP level after the end of bronchoalveolar lavages in our RLL trials is not as harmful to the lung tissue as the aforementioned recruitment maneuvers with an inspiratory pressure of 34 cmH_2O.

De Smet et al. examined the effect of hypercapnia and moderately high tidal volume in IPLs (DE SMET et al., 2007). IPLs were ventilated for 2 h with a high V_T (20 ml/kg) and 20% CO_2. Tidal volume increased IL-6 and TNF-α in bronchoalveolar lavage fluid and perfusate. This stimulation of inflammation is consistent with our findings of increased TNF-α expression in BALF of IPLs ventilated with a V_T of 13.93 ml/kg.

The extraction of the lung-heart bundle from the body, mechanical ventilation, and perfusion of the lung *ex vivo* led to a high concentration of macrophages in the lung tissue which were washed out with the first lavage. Subsequent BALs revealed high amounts of cellular debris and these bronchoalveolar fluids resembled BALFs after IPL treatment with a cell lysing detergent (see Figure 19). This might be due to the fact that time intervals of 5 min between bronchoalveolar lavages were too brief for cellular recruitment from subjacent tissue layers. After the end of lavage

performances, a time interval of 60 min was inserted to analyze the stability of impaired lung function. In controls, this time interval was prolonged to 120 min. After these 2 h, a high amount of macrophages was found to have been recruited to the alveolar epithelium. This cell recruitment finding supports the histological findings and shows the viability of the lung tissue at the end of IPL trials (see Figure 17 and 19).

5.3. Surfactant Administration and Deposition in IPLs

Besides the use of intratracheal catheters, exogenous surfactant can be administered by conducting bronchoscopy, using laryngeal mask airways, or nebulization (BIBAN et al., 2012). Administration forms that are less invasive compared to intratracheal intubation are favored which is why much effort was put into the development of nebulized and aerosolized exogenous surfactant administration forms. The administration of dry powder surfactant aerosols in IPL trials supplied the lungs with highly concentrated surfactant. The dry powder aerosolization was shown to be advantageous compared to nebulization, as no dilution effect via suspension of surfactant in water or saline occurs (POHLMANN et al., 2013).

For the treatment of RDS or ARDS, high amounts of surfactant have to be administered. Consequently, “Dry Powder Inhalers” which are already on the market cannot be used for surfactant administration as they are optimized for single doses in the mg range. Therefore, a new procedure for surfactant administration in IPL and RLL trials, i.e. the continuous powder aerosolizer, was used. The CPA system allows aerosolization of dry surfactant powder and generation of highly concentrated surfactant aerosol (POHLMANN et al., 2013). As administration of aerosolized surfactant to rat lungs *ex vivo* and to rat and lamb lungs *in vivo* was efficient (RAHMEL et al., 2012), the CPA system seems a promising system for noninvasive delivery of surfactant aerosol. The small generated particle sizes that form the wet surfactant aerosol have a mass median aerosol diameter of 3.2 µm and a geometric

standard deviation (GSD) of 2, which enables a promising option for noninvasive ventilator support in preterm neonates or ARDS patients in the future (POHLMANN et al., 2013).

The rate of aerosolized surfactant particle deposition in a specific lung region is influenced by various factors such as the particle concentration that reaches the lung, the breathing frequency and the tidal volume (BROWN et al., 2005). Moreover, the route of breathing through nose or mouth influences the deposition rate of inhaled particles (BROWN et al., 2005). Besides the divergent lung anatomy of humans and rats, the breathing patterns differ immensely. Rats breathe a mean of 102 times/min while humans have a breathing frequency of 12 breath/min (BROWN et al., 2005). The human tidal volume amounts to 625 ml, while rats have a mean V_T of 2.1 ml (BROWN et al., 2005). When comparing the alveolar epithelia of humans and rats, the number of alveoli in the human lung epithelium with around 480 x 106 alveoli is 24 times higher than that of the 20.1 x 106 alveoli that make up the rat lung epithelium (BROWN et al., 2005; HYDE et al., 2004). Another difference in the alveolar epithelium of rats is the uneven allocation of alveoli, of which 59.2% are located in the right lung lobe (HYDE et al., 2004). Despite these vast dimensional differences, with a spherical diameter of 200 µm, human alveoli are only twice the size of rat alveoli, which have a spherical diameter of 100 µm (BROWN et al., 2005; HYDE et al., 2004). If data from inhalation studies shall be transferred from rodent to human, these anatomical and physiological differences might impede predictions about the deposition of aerosolized exogenous surfactant particles.

The generated rSP-C particles in the present study had a MMAD of 1.7 µm (GSD 2.3) (POHLMANN et al., 2013). During aerosol administration, an unequal distribution of surfactant in the lung lobes was assessed macroscopically (see Figure 26). The deposition of particles in the rat lung lobes is not uniform, since the deposition of particles correlates with the lobar volume (ASGHARIAN et al., 2003). It was only towards the end of the administration phase that an opening of all lung lobes was observable. This suggests that a reduction of surface tension in some alveoli leads to an improvement in C_{dyn} which might then result in a further opening of alveoli.

Macroscopically, a faster deposition of exogenous surfactant in the left lung lobe was assessed. These findings are in agreement with investigations of the fate of airborne particles in the rat lung (ASGHARIAN et al., 2003). Asgharian et al. conducted a study in Long-Evans rats and found out that a higher amount of radiolabeled iron chloride particles ranging from 0.9 to 4.2 µm was deposited in the left lung than in each lobe of the right lung. Concerning the right lung, it was observed macroscopically that exogenous surfactant was initially deposited in the lobus cranialis pulmonis dextri and lobus medialis. This finding is inconsistent with the investigations Asgharian et al. in which the highest deposition rate was found in the lobus caudalis pulmonis dextri and the lowest in the lobus accessories. Furthermore, deposition rates in the lobus medialis pulmonis dextri and lobus cranialis were similar (ASGHARIAN et al., 2003).

The deposition of surfactant is directly associated with the tidal volume. The results of impaired lung function in IPLs resulted in a mean V_T of 0.2 ml (bw: 272 ± 36.15 g) at a breathing frequency of 80 min^{-1} at the beginning of surfactant administration. This resulted in a tidal volume of 0.74 ml/kg and an AMV of 58.82 ml/min*kg. The minimum quantity of surfactant deposited in the lung tissue during 250 puffs was 5.5 mg aerosol/IPL. After the administration phase, V_T increased to a mean of 2.1 ml (± 1.46), which accounted for an AMV of 617.65 ml/min*kg. If a linear course for the increase of tidal volume is assumed, a volume of 8455.88 ml/kg was inhaled during an administration period. This led to a mean AMV of 338.24 ml. From RLL trials, a surfactant concentration of 13.2 to 17.5 mg/l is known (in-house data), which results from an aerosolization of approximately 15 - 20 mg surfactant/min and an aerosol flow rate of 1.14 l/min (1.0 l/min sheath flow + 0.14 l/min pulse air). This concentration is also plausible for IPL trials as the same CPA system and equal settings for nebulization were used. If the mean AMV of 338.24 ml is multiplied by the mean surfactant concentration of 15.35 mg/l, a deposited dose of 129.8 mg/kg in IPLs can be assumed over an administration time span of 25 min. When these assumptions are transferred to the RLL model, a deposited dose of about 24 mg/kg bw over an administration time of 15 min can be expected.

Neonates suffering from chronic lung disease (1.95 - 3.8 kg bw) were shown to V_T of 5.15 ± 1.35 ml/kg and a breathing frequency of 55.4 ± 14.2 min^{-1} resulting in a minute volume of 285 ± 148 ml/min*kg (SCHMALISCH et al., 2005). Taking these calculations into account, Pohlmann et al. estimated that during 1 h of dry surfactant powder inhalation about 14 ± 7 to 34 ± 18 mg/kg of aerosolized surfactant would deposit in neonates by inhalation, assuming an aerosol concentration of 8 mg/ml (POHLMANN et al., 2013). By way of comparison, in IPL trials a theoretical amount of 50.26 mg/kg would be deposited in the lung tissue within an hour.

The delivery of dry surfactant powder can be associated with risks of occlusion formation. These occlusions can occur either in the delivery device or in the airways of test animals or patients (POHLMANN et al., 2013). In IPL trials, problems of occlusion formation could also occur in the IPL's chamber lid in the bridge supplying surfactant to the tracheal cannula or in the tracheal cannula itself. Furthermore, dehydration of lung tissue has to be taken into account. Surfactant powder is highly hygroscopic. Sufficient humidification of surfactant is of utmost importance when using a CPA system for surfactant administration (POHLMANN et al., 2013).

Nonetheless, the method of exogenous surfactant administration of rSP-C as an aerosol may be beneficial compared to instillation. In ARDS patients with a Horowitz quotient below 170 mmHg, instillation of rSP-C neither resulted in improvement of oxygenation nor in mortality (SPRAGG et al., 2011). It is assumed that the low efficacy of rSP-C might in part be related to the administration technique. In the preterm lamb model, aerosolized rSP-C could effectively be deposited in both lungs (RAHMEL et al., 2012). Therefore, the method of aerosolized rSP-C administration might be worthwhile. The method for surfactant particle generation in this study was the continuous powder aerosolization system.

5.4. Surfactant Efficacy Testing in IPLs

RSP-C administration was started 1 h after performance of the last bronchoalveolar lavage. This time point represents late treatment in ARDS animal models, whereas early treatment in ARDS animal models would start 10 min after performance of the last saline lavage (HAFNER et al., 1998a). Late treatment reflects a clinical situation of ARDS better than early treatment, since an increased formation of hyaline membranes and infiltration of polymorphonuclear neutrophils can be found in the RLL model after 1 h of saline-impaired lung function (HAFNER et al., 1998a; HAFNER et al., 1998b).

Modifications had to be made in order to efficiently administer surfactant to IPLs (see 3.9). Therefore, different respiratory settings were adjusted to overcome the huge dead pace in the original IPL setting. Among these were a reduction of the respiratory rate during the time of surfactant aerosolization, or a reduction of the respiratory rate during the whole trial. These modifications in the ventilation pattern were thought to be effective in treating the lung tissue more gentle during the alveolar opening process during surfactant treatment, compared to the standard setting (see Figure 25 and Figure 24). As these modified ventilation settings resulted in no long-term improvement of the oxygenation status, the construction of surfactant-aerosol administration in the IPL-2-System was changed. After integration of a new aerosol supply system, administration of dry powder surfactant formulations to IPLs led to significant improvements in tidal volume (see Figure 22) and arterial oxygenation (see Figure 21). Moreover, no significant weight gain during or after the treatment was determined (see Figure 24). It can thus be concluded that exogenous surfactant administration did not result in increased alveolar-capillary permeability.

In control lungs, a slight improvement in lung function was seen which could be explained by slow relief of lung atelectasis and thus an improvement in V_T which results in pO_2 increase (see Figure 28). Only the administration of the surfactant preparation with the highest surfactant protein C content (100% surfactant) led to a significant improvement in dynamic lung compliance and tidal volume at all times

(see Figure 31 and Figure 30). Unlike administration of 100% surfactant, administration of placebo (surfactant preparation without surfactant protein C content) resulted in a clear decrease in V_T of 26% between 10 and 60 min after the end of surfactant administration (see Figure 30). Also, the administration of the surfactant preparation with reduced surfactant protein C content compared to the 100% surfactant preparation (20% surfactant) resulted in a slight decrease in lung function towards the end of IPL trials, which accounted to 14% of reconstituted V_T. These impairments did not affect oxygenation but may offer some insights about the long-term efficacy of different surfactant formulations.

The analysis of pO_2 levels is crucial for the assessment of the efficacy of surfactant batches, which is why special emphasis was placed on the analysis of these data. Even if administration of protein-free placebo surfactant did not result in a significant improvement of lung compliance, a significant increase in oxygenation was observed. This indicates a surface tension lowering effect of protein-free surfactant at the alveolar epithelium. The improvement in arterial oxygenation only resulted in a recovery which was about half of the baseline pO_2 level (see Table 8).

Administration of 20% surfactant resulted in a stable recovery of three quarters of baseline arterial oxygenation levels (see Table 8). Compared to the administration of protein-free surfactant (placebo), administration of 20% surfactant resulted in better oxygenation levels by over 20% at all time points. Even if this improvement was not significantly greater compared to placebo administration (see Figure 28), a low content of protein in surfactant formulations seems to have a stronger influence on surface tension reduction in alveoli than protein-free surfactant alone.

Although tidal volume was significantly improved after administration of placebo or 20% surfactant, arterial oxygenation was only completely recovered after 100% surfactant administration (see Figure 28, Table 8). Moreover, only the 100% surfactant preparation led to a significant further improvement of lung function within 1 h of terminated administration. In contrast to this finding, the oxygenation level of IPLs treated with rSP-C-free or 20% surfactant protein preparation remained stable (see Figure 28).

Our data suggest that the most effective treatment of impaired lung function is achieved by administration of aerosolized surfactant preparations with high rSP-C content. These results are in accordance with findings of superior oxygenation with rSP-C surfactant compared to synthetic surfactant preparations like ALEC® (pumactant) or Exosurf® (colfosceril palmitate) as well as a rSP-C free phospholipid surfactant preparation in a RLL model (HAFNER et al., 1998a). In the same study, rSP-C surfactant was also compared to bovine-derived surfactant preparations like bLES® (bovine lipid extract), Infasurf® (calfactant), and Survanta® (beractant) (HAFNER et al., 1998a). Compared to these surfactant preparations containing SP-B and SP-C, rSP-C surfactant was not found to be significantly inferior when administered at concentrations of 25, 50, or 100 mg/kg bw. The activity of rSP-C in oxygenation was stable at all concentrations (HAFNER et al., 1998a). This shows that a concentration of 25 mg/kg bw is sufficient for significant improvements in lung function. This assumption is in line with our findings of sufficient rSP-C supply at a dose of 20 mg/kg bw in IPLs. Since the surfactant protein preparations Exosurf®, ALEC®, as well as the rSP-C-free phospholipid surfactant showed no increase in activity at doses of 50 or 100 mg/kg bw respectively, it can be hypothesized that administration of higher doses of rSP-C-free placebo surfactant to IPLs would have not resulted in improved oxygenation in IPLs (HAFNER et al., 1998a).

Furthermore, it is widely recognized from clinical findings that protein-free synthetic surfactant preparations are not as effective as protein-containing surfactant formulations in the treatment of RDS. A very recent meta-analysis also showed greater early improvement in the requirement for ventilator support in over 5000 infants treated with animal derived surfactant extracts in comparison to protein-free synthetic surfactant (ARDELL et al., 2015). The same study also showed a lower risk for pneumothorax as well as fewer deaths when RDS patients were treated with protein-containing rather than protein-free surfactant extracts (ARDELL et al., 2015).

5.4.1. Surfactant Efficacy Testing *ex vivo* vs. *in vivo*

Male Sprague-Dawley rats were used in IPL trials to create similar preconditions to the RLL trials. A total of 16 test animals was used in the comparative RLL study, as at least 10 - 12 rats needed to meet the oxygenation criteria (see 3.6). These data were compared to 4 IPL trials in which *ex vivo* lungs received 100% surfactant.

Whereas a total of 8 to 10 blood samples was taken per test animal during an RLL trial, IPL trials allowed constant analysis of pO_2 in the artificial perfusion buffer.

Remarkably, equal pO_2 levels could be reached in the beginning of IPL and RLL trials. This indicates that neither the ventilation technique, whether negative or positive pressure ventilation is performed, nor the height of inspiratory pressure, whether lung were ventilated with -15 or 26 cmH_2O, does cause differences in the uptake of oxygen in alveoli (see Figure 34). Furthermore, one has to notice that IPLs were perfused with artificial buffer that did not contain oxygen-binding hemoglobin. This means that detection of high levels of oxygen is possible in protein containing buffers. The use of different electrodes and measurement techniques did not influence the result of oxygen measurements either.

In comparison to RLL trials, more BALs were performed in IPL trials in order to reach pO_2 levels < 180 mmHg. After performing BALs, oxygen levels in IPL trials did not decrease as much as in RLL trials (see Figure 34). In both models, BALs were performed until pO_2 was < 180 mmHg. This resulted in a further decrease in pO_2 across both models, although this effect was more prominent under positive pressure ventilation. As some animals died during BAL performances in RLL trials, it could be shown that lungs can cope better with a less severe induction of moderate ARDS.

Both models showed an extreme increase in pO_2 after surfactant treatment. The final level of pO_2 in IPL trials was slightly higher compared to administration in RLL trials. In contrast, the magnitude of pO_2 increase was more than twice as large in RLL

compared to IPL trials. This indicates that RLL trials are more effective for the assessment of changes in pO_2 in a secondary surfactant deficiency model.

A common *ex vivo* model in exogenous surfactant research is excised rat lungs (BERMEL et al., 1984; HALL et al., 1992a; HOLM and NOTTER, 1987; IKEGAMI et al., 1977). Ikegami et al. subjected rat lungs that were beforehand excised from the donors, to 20 lavage cycles with 10 ml saline. Afterwards, exogenous surfactant was administered by ultrasonic nebulization or instillation into the trachea of those non-perfused lungs (IKEGAMI et al., 1977). In their study, administration of nebulized surfactant resulted in poorer recovery of quasi-static compliance than instillation of exogenous protein containing surfactant (IKEGAMI et al., 1977). They concluded that nebulization is not an effective method for the treatment of RDS because of insufficient deposition of nebulized exogenous surfactant (IKEGAMI et al., 1977). On the one hand, these findings raise the question whether non-perfused excised rat lungs might be too artificial to represent the physiological circumstances in the alveolar epithelium. On the other hand, it is known from several studies that ultrasonic nebulization of aqueous surfactant preparations is associated with low lung deposition (FINER et al., 2010; SHAH, 2011). Therefore, the low efficacy of nebulized exogenous surfactant found in excised rat lungs may rather be associated with insufficient administration than an inadequate test system.

5.4.2. Limitations and Advantages of *ex vivo* surfactant efficacy testing

Endogenous surfactant depletion with warmed saline lavages is an efficient and frequently used method in animal models of RDS/ARDS. In several mammalian species, reconstitution of lung function with exogenous surfactant treatment was shown to be successful (BERGGREN et al., 1986; KOBAYASHI et al., 1984; WALTHER et al., 1997; WILLSON and NOTTER, 2011). Like every preclinical model, saline lavages as an RDS/ARDS model has its limitations. As such, the model solely represents the status of impaired lung function as it is present in RDS or primary ARDS. Saline lavage secondary surfactant deficient models neither resemble the

complete pathophysiology of ARDS nor the developments of secondary ARDS that can be triggered by sepsis or other pre-existing conditions. An ideal model of ARDS would represent inflammation, an increase in vascular permeability and hypoxemia as the most important criteria which should be met within three to four hours (ZAROGIANNIS and MATALON, 2013). Besides a decrease in oxygenation level in lavaged IPLs, the increased level of TNF-α at the end of IPL trials might indicate a slight inflammation. Moreover, vascular leakage could be seen in increased protein content in BALF at the end of IPL trials.

Other measures, such as the administration of oleic acid do not result in direct surfactant depletion. For instance, in a model of excised rat lungs it could be shown that administration of oleic acid provoked an altered pressure-volume curve and resulted in decreased quasi-static compliance (HALL et al., 1992a). Oleic acid inhibited the endogenous surfactant activity by disrupting the interfacial surfactant layer which is responsible for the generation of very low surface tension during dynamic compression (HALL et al., 1992a). The potential interaction of exogenous surfactant preparations with oleic acid as well as altered endogenous surfactant needs to be taken into consideration.

The aim of this thesis was not to establish a disease model but rather to represent a model of impaired oxygenation. However, if lavaged IPLs are supposed to serve as an ARDS model, they can only reflect the acute onset. Models that might be suitable for late phase ARDS studies could include the bleomycin model, zymosan-induced general inflammation or multiple-hit models in large animals.

Isolated lungs have a significant risk of edema formation during the ventilation and perfusion process. As the pressure gradient along the pulmonary vascular bed is smaller for negative pressure than for positive pressure ventilation (MARINI et al., 2003), pulmonary edema formation might be withhold to some extend in our model of negative-pressure ventilated IPLs. The main risk for edema formation in this study occurred during or after bronchoalveolar lavage performances. Unfortunately, the IPL model is generally not useful for differentiating between interstitial and complete lung edema formation. Also H/E staining did not yield information about edema formation

as the lung tissue was perfused with artificial buffer. This buffer was infiltrated into the lung tissue during edema formation but could not stained due to the lack of dyeable cells in the buffer (see Figure 18).

In comparison excised rat lungs that can be used for investigating the ability of surfactant preparations to lower surface tension, IPLs offer more information. The advantage of IPLs is the analysis of partial oxygen and carbon dioxide pressure in the perfusate. Especially in lung surfactant efficacy testing, the impact of each exogenous surfactant batch on the oxygenation status is of particular interest. Moreover, in IPL trials the impact of exogenous surfactant on the lung function can be analyzed at the time of administration. For analyzing isolated perfused rat lungs, the animal is sacrificed equally as gently as for the procedure of excised rat lungs (see 2.9.3), however the knowledge gained from IPL trials is more relevant.

An advantage of saline secondary deficient models is the use of physiological saline that will most likely not provoke interactions with the treatment. However, in secondary deficiency trials using the RLL model, some animals do not survive repetitive BALs as either blood pH gets too acidic or animals develop lung edema due to impairment of the alveolar-capillary membrane. Both scenarios can also lead to premature decease after completion of pO_2 reduction and also before surfactant administration. After surfactant administration, animals can die because of a radical change in blood pH. Surfactant administration leads to an opening of alveoli and a rapid increase in blood oxygenation. This can cause respiratory alkalosis with a dramatic drop in pCO_2 and a rise in blood pH that cannot be buffered effectively enough in some individuals. In contrast to that, development of respiratory alkalosis is not possible in IPL trials, due to constant pH regulation of the artificial buffer. Therefore, the lung is not in need of blood enrichment with pCO_2.

5.4.3. Use of IPLs for animal welfare in surfactant efficiency testing

Due to anatomical differences of the respiratory tracts, it is questionable whether the rat is the most relevant species for the prediction of effects in the human lung. Nonetheless, rodents are still the most accepted order for efficacy batch testing to date.

The initial estimation that the number of test animals could be reduced by replacing RLL trials with the IPL model because of smaller standard deviations in the measured pO_2 levels could not be upheld. However, the application of IPL trials for efficacy testing of surfactant batches still contributes to a reduction in the use of test animals. Especially the fact that oxygen levels can be monitored throughout the trial without limitations due to volume is a considerable advantage over *in vivo* surfactant batch testing.

All IPLs showed viability 1 h after surfactant administration, which is in stark contrast to the comparative RLL trials in which only half of the animals used survived until the end of the trial. The use of IPLs in surfactant efficacy testing could also contribute to a refinement in animal studies, as for instance gas exchange parameters can be measured continuously. By this, the limiting factor of blood collection is circumvented and a more precise evaluation of the effects of different surfactant batches becomes possible. Another fact that contribute to refinement of animal testing was achieved by the implementation of a measurement unit for constant lung weight. This allowed the assessment of edema formation at a very early stage, compared to whole animal trials. In contrast to the animal trials, in IPLs it was therefore known beforehand if an efficacy of an exogenous surfactant batch was reduced because of potential influence of plasma proteins.

Even if *ex vivo* models are in use of test animals, meaningful data production can be achieved with good reproducibility and a significant reduction in the number of test animals that are used. Moreover, other *ex vivo* trials showed the reproducibility of acute effects from *in vivo* trials (FISCHER et al., 2012). The wide range of respiratory

parameters acquired in IPL trials and especially the measurement of pulmonary resistance and dynamic compliance can be used to localize the anatomic site of a pulmonary effect *ex vivo.*

5.5. Conclusion

The efficacy of exogenous surfactant preparations has to be tested in a batch by batch manner. This thesis set up a surfactant deficiency model using isolated perfused rat lungs and examined the possibility of performing exogenous surfactant efficacy tests *ex vivo*.

Ventilation with moderately low negative inspiratory pressure facilitates the opening of alveoli when endogenous surfactant function is impaired. Artificially perfused rat lungs can be ventilated *ex vivo* with a decreased negative inspiratory pressure for several hours without indications of major impacts on the alveolar-capillary membrane. In the present study, a bronchoalveolar lavage scheme was established. Gentle, repetitive performance of warm saline lavages decreases the pulmonary function to an oxygenation level of moderate ARDS.

Aerosolized exogenous surfactant can be effectively administered to IPLs. An improvement of pulmonary function will be seen after administration to surfactant-deficient IPLs. Moreover, a gradation in the ability to improve oxygenation dependent on SP-C content in the exogenous surfactant preparation is detected.

The limitation of *ex vivo* surfactant testing is that the systemic impact of the treatment cannot be rated. Moreover, the assessable increase in pO_2 after surfactant administration is less than twice the increase found in RLL trials.

For efficacy testing of surfactant batches, an evaluation of the impact of the test preparations on the improvement of systemic oxygenation is the most important parameter. Therefore, an oxygenation-impaired, surfactant-deficient model has to be set up. The study showed that a secondary surfactant-deficiency model can be

reproduced *ex vivo*. Therefore, IPLs might be suitable for efficacy testing of highly concentrated dry surfactant powders.

Finally, in terms of animal welfare, the validation and further development of *ex vivo* models for exogenous surfactant batch testing is highly desirable. In surfactant deficiency models, the impaired lung function of test animals can be associated with suffering or even premature decease. In this context, a validation of the use of *ex vivo* models will contribute to refinement of animal trials, as immediate scarification of test animals is associated with comparatively less suffering than preservation of body functions for several hours. Furthermore, the use of IPLs will lead to an overall reduction in the use of test animals as all lungs reach the end of trial due to stable artificial buffer regulation.

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7. Summary

Dorothee Walter: An Alternative Model for Efficacy Testing of Exogenous Surfactant using the Isolated Perfused Rat Lung (IPL)

Pulmonary surfactant is a surface-active complex that is mainly produced and recycled by alveolar type II cells. Endogenous surfactant consists of lipids (90 - 95%) and proteins (5 - 10%) and is secreted to the alveolar surface. It increases the alveolar stability against collapse and improves uniformity of alveolar inflation by reducing the surface tension of the alveolar air-water interface. Without lung surfactant, gas exchange between blood capillaries and alveoli and thus, normal respiratory function would be considerably impeded or even impossible.

In premature infants, surfactant production can be insufficient due to impaired lung development. Surfactant replacement therapies with exogenous surfactant preparations has been shown to be an efficient treatment in neonates with respiratory distress syndrome (RDS). Moreover, the clinical efficacy of surfactant replacement therapies to treat acute respiratory distress syndrome (ARDS) is discussed.

In terms of regulatory aspects, efficacy of exogenous surfactant batches for the treatment of RDS and ARDS has to be ensured before surfactant administration to patients. Therefore, premature and secondary surfactant deficiency animal models were developed. A frequently used model to test surfactant efficacy is the rat lung lavage (RLL) model in which a secondary surfactant deficiency is provoked by performance of repetitive bronchoalveolar lavages. These studies on anesthetized animals can lead to serious problems like respiratory acidosis causing premature decease of test animals. Therefore, the aim of this thesis was to establish an *ex vivo* model for the testing of exogenous surfactant preparations. Analysis of oxygen levels is the most important parameter in surfactant efficacy testing. Isolated and perfused

rat lungs (IPL) are an ideal test model as permanent arterial oxygen analysis in the perfusate is enabled.

The experiments showed that IPLs can be ventilated with a moderately decreased inspiratory to expiratory ratio -15/-3.6 cmH_2O for up to 240 min without development of pulmonary edema. Moreover, when ventilated with medical oxygen, IPLs reached oxygen levels ≥ 400 mmHg which are in the same range of *in vivo* trials in the RLL model.

The repetitive performance of an average of 6 saline bronchoalveolar lavages (BALs) in IPLs resulted in significant decreases in dynamic lung compliance and tidal volume after the 1^{st} or the 2^{nd} BAL, respectively. This impairment in lung function was accompanied by a decrease of pO_2 < 180 mmHg which corresponds to a status of moderate ARDS and is alike the oxygen levels of lavaged lungs in RLL trials. During BAL performances, an increase in lung weight was noticed which remained stable until the end of IPL trials and therefore did not indicate edema formation. Saline lavages did not result in cytotoxicity in IPLs whereas a significant LDH concentration was found in the lavage fluid after the 5^{th} lavage and at the end of RLL trials. In contrast to cytotoxicity findings, an indication of acute inflammation was found at the end of IPL trials whereas no TNF-α could be found in bronchoalveolar lavage fluids of RLL experiments.

The use of the Continuous Powder Aerosolization (CPA) System enabled the effective administration of aerosolized dry surfactant powder to IPLs. Exogenous surfactant resulted in an improvement of lung function and a complete regeneration of initial oxygen values which applies to the restoration of oxygenation in the *in vivo* situation. Moreover, a gradation in exogenous surfactant efficacies could be seen if preparations with reduced or no protein content were tested. The surfactant preparation with the highest protein content was the most effective one tested. In this study it could be shown that aerosolized rSP-C is effective for the restoration of oxygen saturation one hour after administration.

The results of this thesis indicate that the efficacy of exogenous surfactant batches can be tested *ex vivo*. In terms of animal welfare, the use of IPLs could contribute to reduction and refinement of animal trials. In this context, IPLs are not exposed to premature deceasing and the continuous measurement of the oxygenation status allows precise evaluation of efficacies of saline lavages and exogenous surfactant batches.

8. Zusammenfassung

Dorothee Walter: Ein Alternatives Modell zur Effektivitätsbestimmung von Exogenem Surfactant in der Isoliert Perfundierten Rattenlunge (IPL)

Pulmonales Surfactant ist ein grenzflächenaktiver Komplex, der hauptsächlich von Typ-II-Pneumozyten produziert und recycelt wird. Dieses endogene Tensid besteht aus Lipiden (90 - 95%) und Proteinen (5 - 10%) und wird auf die Oberfläche der Alveolen sezerniert. Surfactant vermindert die Oberflächenspannung an der Alveolar-Kapillar-Membran. Ohne Lungen-Surfactant wäre ein Gasaustausch zwischen den Blutkapillaren und den Alveolen und somit eine normale Atemfunktion deutlich erschwert oder sogar unmöglich.

Bei Frühgeborenen kann die Lungenreifung und damit verbunden eine hinreichende Surfactantproduktion unzureichend sein. Surfactant-therapien mit exogenem Surfactant sind beim Atemnotsyndroms des Neugeborenen (*engl*.: respiratory distress syndrome, RDS) die Standardtherapie. Darüber hinaus wird die klinische Effektivität einer Surfactanttherapie zur Behandlung des akuten progressiven Lungenversagens (*engl*.: acute respiratory distress syndrome, ARDS) diskutiert.

Aus regulatorischer Sicht muss die Wirksamkeit von exogenen Surfactant-Chargen für die Behandlung von RDS und ARDS gewährleistet sein, bevor sie Patienten verabreicht wird. Hierfür werden Tiermodelle mit unreifer Lungenentwicklung oder sogenannte sekundäre Surfactant-Mangel-Tiermodelle eingesetzt. Beim häufig verwendeten Rattenlungen-Lavage (RLL)-Modell, wird ein sekundärer Surfactantmangel durch wiederholte bronchoalveoläre Lavagen (BAL) provoziert. Diese *in vivo* Studien an narkotisierten Tieren können zu ernsthaften Problemen wie respiratorische Azidose und vorzeitigem Versterben der Versuchstiere führen.

Das Ziel dieser Arbeit war es daher, ein *ex vivo* Modell für die Prüfung von exogenen Surfactant-Chargen zu etablieren. Die Analyse des Sauerstoffgehalts stellt hierbei

den wichtigsten Parameter in der Effektivitätstestung dar. Aufgrund der Möglichkeit einer permanenten arteriellen Sauerstoffanalyse im Perfusat, könnte das Modell der isolierten, perfundierten Rattenlunge (IPL) hervorragend als Testmodell genutzt werden.

Die Experimente zeigten, dass IPLs mit einem mäßig verringerten Inspirations- zu Expirationsverhältnis von -15/-3,6 cmH_2O für mindestens 240 min ohne Entwicklung eines Lungenödems beatmet werden können. Wurden IPLs mit medizinischem Sauerstoff beatmet, erreichten sie Sauerstoffgehalte über 400 mmHg, die damit ebenso hoch waren wie die im *in vivo*-Modell (RLL) gemessenen Sauerstoffwerte.

Die wiederholte Lavagierung der IPLs mit physiologischer NaCl-Lösung führte zu einer signifikanten Abnahme der dynamischen Lungencompliance und des Atemvolumens nach der 1. bzw. der 2. bronchoalveolären Lavage (BAL). Diese Beeinträchtigung der Lungenfunktion wurde durch eine Abnahme des pO_2 unter 180 mmHg begleitet. Diese Reduktion entspricht einem moderaten ARDS-Niveau und kann mit den reduzierten Sauerstoffgehalten in RLL-Studien verglichen werden. Während der Durchführung bronchoalveolärer Lavagen wurde ein Anstieg des Lungengewichts verzeichnet, welcher bis Versuchsende stabil blieb und daher nicht auf eine Lungenödembildung schließen lässt. BALs führten in IPLs zu keiner erhöhten Zytotoxizität, während eine signifikant höhere Zytotoxizität in RLL-Versuchen in der Lavagenflüssigkeit der 5. BAL und am Versuchsende festgestellt wurde. Im Gegensatz hierzu zeigte sich ein erhöhtes TNF-α-Level am Ende der IPL-Studien während keine Induktion von TNF-α in Lavagenflüssigkeiten aus RLL-Experimenten gefunden wurde.

Die Verwendung eines Systems zur kontinuierlichen Pulver-Aerosolisierung (*engl*.: Continuous Powder Aerosolization, CPA) ermöglichte eine effektive Zuleitung von aerosolisiertem trockenen Surfactantpulver zu den IPLs. Die Zufuhr von exogenem Surfactant resultierte in einer verbesserten Lungenfunktion und einer vollständigen Regeneration der Anfangssauerstoffwerte, wodurch eine Wiederherstellung der Oxygenierung in der *in vivo* Situation widergespiegelt wird. Darüber hinaus konnte durch die Testung exogener Surfactantformulierungen mit reduziertem oder keinem

Proteingehalt eine Abstufung in der Effektivität nachgewiesen werden. Hierbei weist die Surfactantformulierung mit dem höchsten Proteingehalt die höchste Effektivität auf. Zudem konnte gezeigt werden, dass aerosolisiertes rSP-C auch im IPL-Modell über eine Stunde lang effektiv wirkt.

Die Ergebnisse dieser Arbeit demonstrieren, wie die Wirksamkeit von exogenen Surfactant-Chargen *ex vivo* getestet werden kann. Im Sinne des Tierschutzes kann die Verwendung von IPLs zu einer Verminderung und Verbesserung von Tierversuchen beitragen. Zudem ermöglicht die kontinuierliche Messung des Oxygenierungsstatuses eine genaue Bewertung der Effektivität der Lavagen und der exogenen Surfactant-Chargen.

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