Relationship between Surfactant Alterations and Severity of Disease in Horses with Recurrent Airway Obstruction (RAO)

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ABSTRACT

Pulmonary surfactant is synthesized in the alveoli and lines the respiratory epithelium of the airways. Phospholipids, the main component of surfactant, confer it its ability to lower surface tension and to prevent alveolar collapse. Airway surfactant helps maintain smaller airway patency, improves muco-ciliary clearance, decreases bronchoconstriction, and modulates pulmonary immunity. Surfactant alterations in human asthma are therefore believed to contribute to the severity of airway obstruction.

The goal of our first study was to characterize surfactant phospholipid composition and function in healthy horses, and to investigate the influence of age and bronchoalveolar lavage fluid (BALF) sample characteristics on surfactant. For that purpose, BALF was collected from 17 healthy horses and evaluated for BALF recovery percentage, cell count, and cell differential. BALF was separated into crude surfactant pellets (CSP) and supernatant and was analyzed for phospholipid content, protein content, phospholipid composition, and surface tension. Interestingly, phospholipid (surfactant) content in CSP significantly decreased with age. BALF recovery percentage, nucleated cell count, and cytological profile did not affect surfactant composition or function.

The hypothesis of our second study was that surfactant alterations in RAO-affected horses are related to clinical stage of RAO. The objectives were 1) to compare surfactant phospholipid composition and function between Non-RAO and RAO horses at clinical stages and 2) to investigate relationships between surfactant alterations and variables assessing clinical stage of RAO.

Seven horses with confirmed RAO and seven Non-RAO horses were evaluated in pairs (RAO/Non-RAO) at baseline, during exposure to hay, and post-exposure. Assessments included: clinical scoring, measure of maximal change in pleural pressure (ΔPpl_{max}), airway endoscopy, and BALF cell counts and differentials. Samples were processed and analyzed as described above.

Phospholipid levels in BALF were significantly lower in RAO versus Non-RAO horses, even in the absence of clinical signs. In the group of RAO horses, phospholipid content was significantly lower during exposure versus baseline. Furthermore, exposure to hay led to an increase in the protein versus phospholipid ratio in BALF from RAO horses. No significant differences were found in BALF protein content, phospholipid composition, or surface tension between or within groups of horses. Phosphatidylglycerol percentage had a tendency to be lower in RAO horses with higher clinical scores. Supernatant protein content was related to BALF neutrophilia in RAO crisis and overall ΔPpl_{max} .

In conclusion, our study demonstrated that surfactant alterations in RAO horses are present in remission and are exacerbated following exposure to hay. It is conceivable that a lower amount of surfactant in bronchioli of RAO horses may contribute to the horses' propensity to develop airway obstruction, mucous accumulation, and bronchial hyperresponsiveness. This may be exacerbated during crisis by a relatively higher protein versus phospholipid ratio. Furthermore, a progressive decrease of surfactant levels in older horses may contribute to a worsening of clinical signs in older RAO-affected horses.

Attribution

Several colleagues and coworkers aided in the writing and research behind several of the chapters of this dissertation. A brief description of their background and their contributions are included here.

Prof. Virginia Buechner-Maxwell - Ph.D. (Department of Large Animal Clinical Sciences, Virginia Tech) is the primary Advisor and Committee Chair.

Chapter I, Section 1: Role of Lung Surfactant in Respiratory Disease: Current Knowledge in Large Animal Medicine

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Duncan Hite - MD (Department of Internal Medicine, Wake Forest University School of Medicine) was a member of the PhD committee. His mentorship aided in the writing and editing of this article.

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LIST OF ACRONYMS AND ABBREVIATIONS

Ach	acetylcholine
ALI	acute lung injury
ANOVA	analysis of variance
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
BWB	Belgian white blue
cAMP	cyclic adenosine monophosphate
CapS	capillary surfactometer
CBC	complete blood cell count
CBS	captive bubble surfactometer
cGMP	cyclic guanosine monophosphate
CLSE	calf lung surfactant extract
COPD	chronic obstructive pulmonary disease
CPAP	continuous positive airway pressure
CS	clinical score
CSD	constraint sessile drop
DPPC	dipalmitoylphosphatidylcholine
eCLCA1	equine soluble secreted glycoprotein
eNANC	excitatory non adrenergic non cholinergic
EqNARDS	equine neonatal acute respiratory distress syndrome
FA	fatty acids
FLM	fetal lung maturity testing
GRR	ratio of oxidized to reduced glutathione
GSH	reduced form of glutathione
HPLC	high performance liquid chromatography
iNANC	inhibitory non adrenergic non cholinergic
IV	intravenous
L/S	lecithin to sphingomyelin ratio
LA	large aggregates

LFT	lung function testing
LOSA	loss of surface activity
LPC	lysophosphatidylcholine
LWB	Langmuir Wilhelmy balance
MOF	multiple organ failure
MS	mucous score
n	number
NCC	nucleated cell count
NERDS	neonatal equine respiratory distress syndrome
NF-κB	nuclear factor kappa B
NRDS	neonatal respiratory distress syndrome
p	significance level
PB	phosphatidylbutanol
PBS	pulsating bubble surfactometer
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL CSP	phospholipid content (µg/ml BALF) in crude surfactant pellet
PL Supe	phospholipid content (µg/ml BALF) in supernatant
PL Total	total phospholipid content (µg/ml BALF) in cell-free bronchoalveolar lavage fluid
PL	phospholipid
Prot CSP	protein content (µg/ml BALF) in crude surfactant pellet
Prot Supe	protein content (µg/ml BALF) in supernatant
Prot Total	total protein content (µg/ml BALF) in cell-free bronchoalveolar lavage fluid
PS	phosphatidylserine
r	correlation coefficient
r^2	coefficient of determination
RAO	recurrent airway obstruction
RDS	respiratory distress syndrome
SA	small aggregates

SIRS	systemic inflammatory response syndrome
SP-A	surfactant protein A
SPAOPD	summer pasture associated obstructive pulmonary disease
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D
$sPLA_2$	secretory phospholipase A_2
SPM	sphingomyelin
SurfA	surface activity
TLC	thin layer chromatography
TPPL	total protein versus total phospholipid ratio
TRALI	transfusion related acute lung injury
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine
γ_{min}	minimal surface tension (mN/m)
ΔPpl_{max}	maximal change in pleural pressure during tidal breathing

Hypothesis and objectives

Lung surfactant is a complex mixture of phospholipids, neutral lipids and surfactant specific proteins lining the epithelial surface of the lung.[1] Lung surfactant plays a vital role in the alveoli by lowering surface tension and preventing alveolar collapse.[2] The clinical importance of surfactant is evident from the significant impact that exogenous surfactant administration has had on preterm infant mortality, as well as the contribution of surfactant alterations to the respiratory failure associated with acute respiratory distress syndrome.[3]

Airway surfactant promotes mucociliary clearance, opposes airway edema, reduces the sensitivity and overall magnitude of the bronchoconstrictive response and prevents collapse of terminal airways.[4-7] Surfactant also assumes important immunomodulatory function.[8] Considering these surfactant functions, it is not surprising that surfactant dysfunction has been implicated in the development of airway diseases such as asthma.

Human asthma and equine recurrent airway obstruction (RAO) are characterized by chronic airway inflammation, reversible airway obstruction and bronchial hyperresponsiveness.[9] Both diseases can be controlled with environmental management and by administration of corticosteroids and bronchodilators. Increased levels of IgE in BALF and serum from RAO-affected horses and a Th2-type cytokine profile are further findings that suggest that RAO shares certain similarities with human asthma.[10,11]

A growing body of experimental evidence indicates that surfactant dysfunction may contribute to the airway obstruction in asthma.[12] Several structural and animal models showed the importance of surfactant in maintaining airflow within conducting airways.[13-15] Surfactant dysfunction has been identified in bronchoalveolar lavage fluid (BALF) from asthmatic patients after endobronchial allergen challenge and in sputum from stable asthmatic patients.[16-19] Surfactant composition in asthmatic patients after allergen challenge is significantly different when compared to healthy subjects.[16,18,20] Changes in composition include a decrease in the large surfactant aggregates (considered as surface active) in comparison to small surfactant aggregates (considered as less surface active) and an increase in the ratio between phosphatidylcholine (PC) and phosphatidylglycerol (PG). Leakage of plasma proteins into the airways appears to play a fundamental role in surfactant inactivation.[16,20] Hydrolysis of surfactant phospholipids via

phospholipases further contributes to surfactant inactivation.[18] Despite these findings it is not clear to what extent surfactant dysfunction relates to the severity of clinical signs. It is also not known if changes in surfactant composition render animals more prone to airway disease or if changes identified in asthma are strictly a result of airway inflammation. Therefore, it is likely that surfactant alterations that have been described in human asthmatics may also be present in RAO affected horses.

Only a few reports exist regarding the changes in surfactant composition associated with airway disease in horses.[21-25] Increased surfactant surface tension is reported in exercising horses.[23] Changes in surfactant composition also occur in horses transported for a prolonged period of time and horses undergoing general anesthesia.[21,22,26] Surfactant alterations described in RAO horses with clinical disease include decreased phospholipid content, alterations in surfactant aggregate ratio, tendency for increased surface tension, and increase in PC/PG ratio.[25] However, surfactant composition and function has not been reported in normal horses or RAO horses during remission, nor have changes that occur with short term exposure to challenge environment, and subsequent recovery.

Our first goal was to evaluate surfactant composition and function in a group of healthy horses and to investigate the influence of age and BALF sample characteristics on surfactant variables.

Our second goal was to evaluate surfactant composition and function in Non-RAO and RAO horses at different disease stages. Our hypothesis was that surfactant alterations in RAO horses are related to disease severity. Our objectives were 1) to compare surfactant composition and function in RAO horses with that of Non-RAO horses, and 2) to evaluate possible relationships between surfactant alterations and RAO disease severity.

CHAPTER I
LITERATURE REVIEW

Section 1:
Role of Lung surfactant in Respiratory Disease:
Current Knowledge in Large Animal Medicine

Undine Christmann, Virginia Buechner-Maxwell, Sharon Witonsky, Duncan Hite

ABSTRACT

Lung surfactant is produced by type II alveolar cells as a mixture of phospholipids, surfactant proteins, and neutral lipids. Surfactant lowers alveolar surface tension and is crucial for the prevention of alveolar collapse. In addition, surfactant contributes to smaller airway patency and improves muco-ciliary clearance. Surfactant specific proteins are part of the innate immune defense mechanisms of the lung. Lung surfactant alterations have been described in a number of respiratory diseases. Surfactant deficiency (quantitative deficit of surfactant) in premature animals causes neonatal respiratory distress syndrome (NRDS). Surfactant dysfunction (qualitative changes in surfactant) has been implicated in the pathophysiology of acute respiratory distress syndrome (ARDS) and asthma. Analysis of surfactant from amniotic fluid allows assessment of fetal lung maturity (FLM) in the human fetus and exogenous surfactant replacement therapy is part of the standard care in premature human infants. In contrast to human medicine, use and success of FLM testing or surfactant replacement therapy remain limited in veterinary medicine. Lung surfactant has been studied in large animals models of human disease. However, only a few reports exist on lung surfactant alterations in naturally occurring respiratory disease in large animals. This article gives a general review on the role of lung surfactant in respiratory disease followed by an overview of our current knowledge on surfactant in large animal veterinary medicine.

This article (Chapter I, Section 1) has been submitted for publication to the journal of Veterinary Internal Medicine (JVIM). If it is accepted for publication, copyright permission will be requested from the journal for the entire document.

List of abbreviations and acronyms

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
BWB	Belgian white blue
CPAP	continuous positive airway pressure
DPPC	dipalmitoylphosphatidylcholine
EqNARDS	equine neonatal acute respiratory distress syndrome
FA	fatty acids
FLM	fetal lung maturity testing
L/S	lecithin to sphingomyelin ratio
LA	large aggregates
LPC	lysophosphatidylcholine
MOF	multiple organ failure
NERDS	neonatal equine respiratory distress syndrome
NRDS	neonatal respiratory distress syndrome
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
RDS	respiratory distress syndrome
SA	small aggregates
SIRS	systemic inflammatory response syndrome
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D
$sPLA_2$	secretory phospholipase A_2
SPM	sphingomyelin

TRALI transfusion related acute lung injury

Introduction

Lung surfactant plays a crucial role in the prevention of alveolar collapse through its ability to reduce surface tension.[2,3,27] Lung surfactant deficiency due to pulmonary immaturity leads to neonatal respiratory distress syndrome (NRDS), which was a major cause of mortality in premature human infants prior to 1990. The relationship between surface tension and elastic forces of the lung was first demonstrated by Kurt von Neergaard in 1929.[28] In 1958 Pattle described the properties, function, and origin of the alveolar lining layer.[29] Shortly thereafter the link between surfactant deficiency and human NRDS was established.[30] A respiratory distress syndrome in foals was reported several years later and foal surfactant was characterized by some of the same researchers that first described it in human medicine.[31-33] Whereas fetal lung maturity testing and exogenous surfactant therapy significantly enhanced survival in premature infants,[34] their use and success in veterinary medicine has remained limited.[35,36]

Since the original discovery of surfactant, knowledge about its composition and function has expanded significantly. Surfactant specific proteins were characterized and attributed important biophysical and immunomodulatory properties.[37] The role of surfactant dysfunction (the loss of its ability to lower surface tension) was described in a number of alveolar and airway diseases.[2,27] In addition, it was discovered that surfactant composition and function vary according to the type of animal species, degree of lung development, and physiological needs.[38,39] Only a few studies exist that evaluated surfactant alterations in naturally occurring diseases in veterinary medicine.[21-26,40,41] In this article, an overview of surfactant function and composition will be presented, with an emphasis on information relating to large animal medicine.

Surfactant composition

Lung surfactant is produced by alveolar type II cells and consists of a mixture of phospholipids and proteins.[42] Lung surfactant recovered from bronchoalveolar lavage fluid (BALF) is composed of approximately 80-85% phospholipids, 5-10% proteins, and 5-10% other lipids (see Table I.1.1.).[2,27]

Phospholipid composition

Phospholipids (PLs) are the main components that confer surfactant its ability to lower surface tension. PL are molecules with an amphipathic structure: two hydrophobic, non-polar fatty acid (FA) chains and a hydrophilic, polar head group are attached to a 3-carbon glycerol backbone (Figure I.1.1).[3] PL class is determined by the type of head group present which is either zwitterionic (with a positive and a negative charge) or anionic (with a negative charge).[3] Each PL class can be further subdivided into different molecular species according to the specific FA chains (as determined by length and degree of saturation) which are attached.[39,43] Lung surfactant contains several major PL classes: phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SPM).[2,27] PC is the most abundant PL and contains predominantly saturated molecular species. Dipalmitoyl-PC (DPPC) accounts for at least 50% of PC molecular species and is a critically important component for achieving maximum reduction of surface tension. PG is the second most abundant PL in surfactant and is used as an indicator of lung maturity in human neonates (see Figure I.1.2.).[44] PI accounts for a small percentage of surfactant PL, and is considered a marker of lung immaturity.[44] PG and PI are anionic PL's and may substitute for each other in maintaining certain surfactant functions. PE, PS, and LPC are present in relatively low concentrations in lung surfactant. Their increase can indicate ongoing inflammation and cellular injury.[2]

Protein composition

A total of 4 surfactant proteins have been identified thus far (SP-A, SP-B, SP-C, and SP-D), and are classified by their relative affinity for water (hydrophilic) versus lipids (hydrophobic).[37] Alveolar type II cells synthesize all 4 surfactant proteins.[8,37] SP-A, SP-B, and SP-D are furthermore produced by airway cells such as Clara cells and submucosal cells.[8] In addition, SP-A and SP-D have been described in several extra-pulmonary locations.[8]

Hydrophilic surfactant proteins SP-A and SP-D

The main function of SP-A and SP-D is to participate in innate pulmonary immune defenses.[8] SP-A and SP-D are members of the collectin protein family, which share a common

structure including: an amino (N)-terminal, a collagen-like domain, a neck region, and a carbohydrate recognition domain (CRD).[37,45] SP-A, the most abundant surfactant protein, consists of an octadecamer assembled into a bouquet-like structure (Figure I.1.3.).[8,37,45] SP-D, which is less lipid bound compared to the other surfactant proteins, is composed of a dodecamer comprised of four trimers forming a cruciform structure (Figure I.1.3.).[45,46]

Hydrophobic surfactant proteins SP-B and SP-C

SP-B and SP-C are essential for biophysical functions of surfactant.[37] SP-B is the only surfactant protein vital for postnatal lung function.[47] Complete deficiency of SP-B in mice and humans results in lethal neonatal RDS.[48,49] SP-B is present in the alveoli in form of a dimer with amphipathic properties that allow interaction with surfactant PL (Figure I.1.3.).[37,47] SP-C is the smallest and most hydrophobic surfactant protein.[37,47] It contains a transmembrane α-helix and 2 palmitoyl groups that interact with PL side chains (Figure I.1.3.).[47]

Other lipids

Surfactant contains a small percentage of neutral lipids that are generally comprised of cholesterol, cholesterol esters, diglycerides, triglycerides, and free fatty acids.[3,42] The roles of these components for surfactant function have not been fully characterized.[50,51]

Lung surfactant functions

Biophysical functions

The best-known function of surfactant is its ability to reduce surface tension. Surface tension is defined as the force acting across an imaginary line of 1 cm in length on the surface of a liquid.[52] Attractive forces between adjacent molecules of liquid are stronger than those between the liquid and overlaying gas. Surface tension of saline is approximately 70 mN/m whereas surface tension of surfactant at the end of expiration approaches values below 1mN/m.[3] The amphipathic properties of lung surfactant allow its alignment at the air-liquid interface. Surfactant intermolecular repulsive forces act by opposing attractive forces between molecules at the liquid surface. The presence of lung surfactant in the alveoli therefore reduces surface tension.

Lung surfactant allows an adaptation of surface tension during the respiratory cycle. The law of LaPlace states that the pressure difference (ΔP) across a spherical surface (i.e. alveoli) depends on the ratio between surface tension (σ) and the radius (r) of the sphere.[2,3,27]

$$\Delta P = 2\ \sigma/r$$

This equation serves to illustrate the relationship between the radius of a sphere and the change in pressure required to dilate that sphere. A constant surface tension in conjunction with a small radius would lead to a high pressure difference across the sphere. At the level of the alveoli, this would translate to an increased tendency for collapse of smaller alveoli during expiration.[2,3,27] The presence of lung surfactant allows pressure differences across the alveolus to remain constant throughout the respiratory cycle. At the end of expiration the surfactant film is enriched in DPPC inducing surface tensions near zero thus preventing alveolar collapse (for additional information see 'Surfactant metabolism and surfactant film formation'). Lung surfactant supports alveolar stability during the respiratory cycle, allows alveoli of different sizes to function with equal efficiency, increases lung compliance, and reduces the work of breathing. The presence of lung surfactant also helps maintain the gas exchange area of the lung. Furthermore, lung surfactant counteracts edema formation by balancing hydrostatic forces.[2,27]

The potential role of lung surfactant in maintaining patency of terminal bronchi was first described in 1970 by Macklem et al.[53] Surfactant at the level of the airways is most likely derived from alveolar material.[43] Low surfactant surface tension promotes smaller airway patency, counteracts fluid accumulation, and prevents edema formation.[53-55] Surfactant forms a non-specific barrier against the adhesion and invasion of microorganisms into the lung.[6] Furthermore, airway surfactant was shown to improve muco-ciliary clearance,[56] and to reduce bronchoconstriction in response to inhaled allergens.[6] The latter function was explained by a potential masking of irritant receptors within bronchi.[6] A recent study suggested the role of surfactant as a smooth muscle relaxant.[7]

Host defense functions

Host defense functions of surfactant are primarily assured by SP-A and SP-D. Nevertheless, surfactant PLs also influence pulmonary immune function.

Surfactant proteins SP-A and SP-D

In recent years the role of SP-A and SP-D in pulmonary immune defense mechanisms has been described (see Figure I.1.4.).[8,57] As members of the collectin protein family, SP-A and SP-D opsonize bacteria, viruses, fungi, and allergens by attaching to various binding motifs on their target via the CRD.[58-64] CRD are oriented in a manner that confers unique carbohydrate recognition specificities.[8,59] SP-A and SP-D facilitate phagocytosis of pathogens by at least 3 different mechanisms: opsonization of pathogens (as described earlier), ligand-activation of immune cells, and upregulation of cell receptors involved in microbial recognition.[8] Immune cells involved in pathogen uptake include alveolar macrophages, monocytes, neutrophils, and dendritic cells. SP-A and SP-D modulate production of inflammatory mediators.[65] The type of effect (upregulation or downregulation of immune response) depends on the pathogen or stimulus present, the duration of exposure, the state of activation of the immune cell, and the type of cell and receptor engaged.[8,65] Collectins also have a direct microbicidal activity against bacteria or fungi without the presence of immune effector cells.[8,66] The mechanisms leading to microbial killing have not been fully characterized.

Surfactant proteins promote phagocytosis of apoptotic cells.[67] SP-A and SP-D promote the uptake of apoptotic cells by alveolar macrophages.[67] In addition, SP-A enhances the induction of an anti-inflammatory response by phagocytes following uptake of apoptotic cells.[68]

Surfactant phospholipids

In general, surfactant PLs tend to downregulate and suppress immune cell function.[69] In addition, surfactant PLs inhibit the production of reactive oxygen intermediates by various cells including alveolar macrophages and peripheral blood neutrophils.[70] PG and SP-A contribute to the inhibition of the group IIA secretory A_2 phospholipase ($sPLA_2$) synthesis by alveolar macrophages.[71] PLs also slow the release of proinflammatory mediators and inhibit the activation of nuclear factor κB and the L-selectin-induced signal transduction.[2,12]

Surfactant metabolism and surfactant film formation

Surfactant PL and proteins are synthesized in the endoplasmic reticulum of type II alveolar cells (Figure I.1.5.).[42] In addition, SP-A, SP-B, and SP-D are produced by Clara cells

and submucosal cells.[8] Phospholipids and surfactant proteins are processed through the Golgi apparatus and packaged into lamellar bodies (intracellular storage organelles).[3,42] Surfactant is exocytosed from these structures into the alveolar hypophase (a liquid layer between air and lung epithelium) where it exists in form of heterogeneous PL-rich aggregates including tubular myelin.[72] Tubular myelin, a crosshatched network of PL bilayers, provides a continuous adsorption of PL to the surfactant film at the air-liquid interface. The majority of surfactant is recycled by endocytosis and reincorporated into metabolic pathways.[72] A small part of surfactant (10-15%) is degraded by alveolar macrophages and 2 to 5% of surfactant is eliminated towards the airways through its role in enhancement of muco-ciliary clearance of pathogens and particles.[2,3] Surfactant recovered from BALF via ultracentrifugation contains 2 different aggregate forms: large aggregates (LA) and small aggregates (SA).[73,74] LA have been demonstrated to be highly surface-active and to contain the hydrophobic surfactant proteins along with SP-A and specialized PL structures (i.e. lamellar bodies and tubular myelin). SA contain SP-D and are less surface active despite a PL composition that is similar to LA. SA typically represent surfactant that is being removed from the air-liquid interface for recycling or has been injured during inflammation.[75] The LA to SA ratio is often altered in disease states characterized by lung inflammation.[76]

The surfactant film at the air-liquid interface is subjected to compression and expansion during breathing.[3] The following concept has been proposed for surfactant film behavior: PLs with unsaturated FA chains are squeezed out of the surfactant film during expiration (compression) leading to enrichment in DPPC. DPPC films can be tightly compressed which allows achievement of very low surface tensions at the end of expiration and thus prevents alveolar collapse. During inspiration (expansion) expelled surfactant components are reincorporated into the surface active film and provide enhanced surfactant spreading. Although the ability to maximally lower surface tension is attributable to the presence of DPPC in surfactant films, proper surfactant function, which is highly dynamic in vivo, could not be achieved without the action of other lipids and surfactant proteins.[27] PLs with unsaturated or shorter FA chains introduce fluidity into surfactant films, and allow fast surfactant adsorption and re-spreading at the air-liquid interface.[3] Anionic PLs (PG and PI) enhance surfactant film adsorption, and film stabilization during the respiratory cycle.[77] This function has been attributed to their ability to interact with hydrophobic SP-B.[78] Surfactant proteins SP-B and SP-C along

with SP-A are essential for packaging of surfactant PL in lamellar bodies, organization of tubular myelin, and ultimately formation of an efficient surfactant film at the air-liquid interface.[79,80]

Surfactant abnormalities in human respiratory diseases

Mechanisms of lung surfactant abnormalities

Increased surface tension of surfactant can be caused by surfactant deficiency (a quantitative deficit of surfactant) or surfactant dysfunction (qualitative changes in surfactant).[3] Surfactant deficiency may be the result of an immaturity or injury to type II alveolar cells leading to insufficient surfactant synthesis, storage, or release. Surfactant dysfunction or loss of surface activity can be caused by a variety of surfactant inhibitors or by the degradation of surfactant components.[3] (Table I.1.2.) Surfactant inhibitors include plasma proteins (i.e. albumin, fibrinogen or fibrin), hemoglobin, cellular lipids (i.e. non-saturated phospholipids, lysophospholipids, cholesterol, FA), and meconium. Some of the components mentioned are found in low concentrations in normal surfactant. In contrast, inhibitors accumulate in the alveoli (following damage to the alveolocapillary membrane, pulmonary inflammation, or aspiration) and hinder a normal surfactant film spreading at the air-liquid interface.[53-55,81] Degradation of surfactant components occurs via the action of proteases, phospholipases or reactive oxygen species liberated during pulmonary inflammation. Neutrophil elastase is one example of an inflammatory protease that has been shown to alter surface activity of surfactant by inactivating surfactant proteins.[82] Phospholipases hydrolyze phospholipids and produce FA and lysophospholipids that interfere with surfactant film fluidity.[83,84] Decreased levels of SP-B, altered surfactant aggregate formation and metabolism are additional mechanisms that have been proposed for surfactant dysfunction.[3]

Surfactant alterations have been implicated in the pathophysiology of a number of alveolar and airway diseases. We have selected a few specific respiratory diseases to illustrate the role of surfactant in respiratory pathophysiology: NRDS as a classic disease of surfactant *deficiency*, SP-B deficiency as a lethal *congenital mutation* of surfactant, acute respiratory distress syndrome (ARDS) and asthma as examples of both *alveolar and airway* surfactant *injury and depletion*. Surfactant alterations have been described in a much larger number of respiratory diseases, but these will not be addressed within this review.[2,27]

Surfactant deficiency: Neonatal respiratory distress syndrome (NRDS)

It is well established that surfactant deficiency plays a central role in the pathophysiology of neonatal respiratory distress syndrome (NRDS). This fact has led to the development of fetal lung maturity testing (FLM) and surfactant replacement therapy in premature infants, and each of these developments have significantly advanced neonatal care.

Neonatal respiratory distress syndrome (NRDS) affects primarily premature human infants and is caused by lung immaturity.[34,85] Maturation of alveolar type II cells and surfactant synthesis in the fetus is a crucial part of lung development and occurs during the last trimester of gestation. Incidence of NRDS is inversely related to gestational age of affected patients.[44,86] For example, in human medicine 60% of infants develop NRDS at less than 28 weeks, 15-20% at 32 to 36 weeks, and less than 5% at over 37 weeks of gestation.[3] Predisposing factors for NRDS include prematurity, prolonged sedation of the mother, fetal head injury, aspiration of blood or amniotic fluid, maternal diabetes, ceasarian-section, as well as intrauterine hypoxia.[34,87]

Premature newborns with surfactant deficiency develop respiratory distress and cyanosis shortly after birth.[3,85] Patient status may improve with oxygen therapy or may require mechanical ventilation.[85] An alveolar or severe interstitial lung pattern is revealed on radiographs of affected patients.[3] High alveolar surface tension is responsible for extensive alveolar collapse leading to pulmonary atelectasis, ventilation-perfusion mismatching, hypoxemia, hypercapnia, and respiratory acidosis.[3] Epithelial and endothelial damage at the alveolo-capillary junction results in plasma leakage, accumulation of fibrin, inflammatory cells, necrotic cell debris, and formation of hyaline membranes. Disease progression may induce further surfactant alterations due to inhibition or degradation of surfactant.[3] Surfactant alterations described in human NRDS include: increased surface tension, decreased concentration of total PL, DPPC, PG, and SP-A.[2,27]

Prevention of premature birth is the cornerstone of NRDS prophylaxis in human medicine. Development of FLM tests by amniotic fluid analysis was a major step in the management of high risk pregnancies (see 'Fetal lung maturity testing').[44,86] Prevention or treatment of NRDS should begin prior to delivery.[85] In pregnancies with threatened preterm labor before 35 weeks of gestation administration of corticosteroids (i.e. betamethasone) at 48 to

72 hours before delivery is recommended to hasten FLM.[85,86] A short-term delay of birth to enable corticosteroids to take effect may be achieved by use of tocolytic drugs.[85] Treatment of infants affected by NRDS includes surfactant replacement therapy (see 'Surfactant replacement therapy'), oxygen supplementation, continuous positive airway pressure (CPAP), and supportive care.[85,86]

Congenital Surfactant mutations: Hereditary SP-B deficiency

SP-B deficiency is a rare, lethal disease described in newborn mice and humans.[48,49] Clinical signs develop within 24-48 hours of birth and are similar to those reported for NRDS.[49] In contrast to classical NRDS, patients affected by hereditary SP-B deficiency are full-term and do not respond to exogenous surfactant replacement. Lung transplantation has been described as the only treatment option.[88] The most common form of SP-B deficiency is autosomal recessive. Mice with a targeted gene deletion for SP-B develop RDS soon after birth.[89] Ultrastructural examination of lung tissue reveals an accumulation of disorganized multivesicular bodies and absence of lamellar bodies in type II alveolar cells.[90] An accumulation of mis-processed SP-C in alveolar cells and amniotic fluid are further characteristics of SP-B deficiency.[79] These findings emphasize the role that SP-B plays in surfactant processing in type II alveolar cells, surface activity in the alveoli, and lung function in the neonate.[91]

Surfactant Injury and Depletion:

Acute respiratory distress syndrome (ARDS): alveolar disease

Acute respiratory distress syndrome (ARDS), previously also known as *Adult* RDS, is characterized by a severe life-threatening inflammatory event within the lung.[92,93] ARDS is known to cause extensive surfactant dysfunction and depletion.[76,94,95] ARDS affects patients of all age groups. Inciting causes for ARDS may be a direct injury to the pulmonary parenchyma (direct ARDS) or may be the result of the systemic inflammatory response syndrome (SIRS) which when carried to the lung via the pulmonary vasculature results in injury of the alveolar endothelium and epithelium (indirect ARDS).[93,94] Examples of direct and indirect ARDS include: pneumonia, inhaled or aspirated noxious agents, sepsis, blood transfusions (TRALI), pancreatitis, and traumatic injuries with multiple fractures and/or lung contusion.[3]

The definition of ARDS is clinical and includes the following: acute in onset, significant hypoxemia as defined by a P/F ratio (ratio of arterial oxygenation to inspired oxygen PaO_2/FiO_2) of less than 200mmHg, bilateral infiltrates on chest radiographs, and no evidence of left atrial hypertension.[96] Sepsis and multi-organ failure (MOF) are often associated with ARDS.[93] The acute stage of ARDS is characterized by damage to capillary alveolar membrane, influx of protein-rich edema fluid into the alveolus, rise in pulmonary vascular resistance and severe ventilation/perfusion abnormalities. Surfactant dysfunction in ARDS patients is likely caused by a combination of secondary surfactant deficiency, surfactant inhibition and degradation. Surfactant alterations described in ARDS patients include: high surface tension;[97-100] reduced levels of large surfactant aggregates;[97-99] decreased PC and PG;[97-102] increased PI;[97-99,101,102] increased PE; [97,99,102] increased SPM;[97-100,102] and decreased SP-A and SP-B.[97,99]

Treatment of ARDS consists of oxygen supplementation and ventilatory support, fluid and hemodynamic management, and antimicrobial therapy.[93] Surfactant administration remains an experimental treatment in patients with ARDS with no commercially available products having FDA approval for an ARDS indication. A recent meta-analysis of exogenous surfactant treatment for ARDS demonstrated that even though this treatment had a tendency to improve patient oxygenation status, it did not improve mortality.[95] Many factors can affect the outcome of surfactant trials: nature of lung injury, type of surfactant preparation used, dose and delivery route, mode of mechanical ventilation, and timing/duration of administration.[103] Unlike NRDS where early surfactant administration is treating a pure surfactant deficiency and effectively prevents lung inflammation, administered surfactant in ARDS patients is likely inactivated rapidly due to the pre-existing severe inflammatory process.

Asthma: airway disease

Allergic asthma is a reversible airway obstruction characterized by airway hyperresponsiveness, airway inflammation, and mucus plugging.[104] Clinical signs associated with asthma are wheezing (exacerbated during expiration), dyspnea, coughing, use of accessory muscle of respiration, and evidence of airflow obstruction. The early stage of asthma develops within minutes of exposure to inhaled allergens and is characterized by mast cell degranulation, release of histamine, leukotrienes, and other inflammatory mediators. The late asthmatic

response leads to more severe symptoms and a predominantly eosinophil mediated inflammatory response.

Over the past two decades, there has been an increasing interest in the possible contribution of surfactant dysfunction to airway obstruction and inflammation in asthma.[16-20,105] Alterations in airway surfactant have the potential to decrease smaller airway patency, decrease muco-ciliary clearance, increase airway edema, contribute to bronchoconstrictive response to inhaled allergens, and affect pulmonary immune responses. Surfactant dysfunction was identified in BALF from asthmatic patients after endobronchial allergen challenge and in sputum from stable asthmatic patients.[16-20,105] Surfactant alterations associated with asthma include high surface tension, altered surfactant aggregate ratio (decreased LA/SA), and changes in surfactant PL composition (decreased PC/PG).[16,18,20,106] Causes implicated in surfactant alterations described in asthmatic patients include: non-specific protein infiltration into the airways,[16,20,105] release of specific inflammatory proteins (i.e. eosinophilic cationic protein),[107] and increased levels of $sPLA_2$, which has the ability to hydrolyze surfactant phospholipids.[18,106] Studies on alterations in surfactant proteins SP-A and SP-D in asthmatic patients have yielded conflicting results.[108-110] Two small pilot studies have evaluated the effect of surfactant therapy in asthmatic patients and reported some improvement in airflow obstruction.[111,112]

Fetal lung maturity testing

In human medicine, fetal lung maturity (FLM) testing is a routine procedure in high risk pregnancies.[44] Fetal lung secretions are washed out to the amniotic fluid. Consequently, analysis of amniotic fluid can provide information related to fetal lung surfactant composition and maturity.[44] Fetal surfactant composition undergoes significant changes towards the end of gestation. An example of lung surfactant PL changes in human amniotic fluid during pregnancy is depicted in Figure I.1.2. Amniotic fluid collected via amniocentesis is the specimen of choice for FLM testing.[113] Samples may also be obtained by transvaginal puncture of the bulging membranes or vaginal pool samples. Samples should be free of contamination by blood or meconium since these can significantly alter test results.

The first test developed for FLM testing was measurement of the lecithin (older term for PC) to sphingomyelin ratio (L/S ratio).[44,86,114] In uncomplicated pregnancies the L/S ratio reaches

values of 2 by 35 weeks of gestational age. NRDS is considered unlikely to develop in infants if a L/S ratio of 2 has been achieved. L/S ratio is considered the 'gold standard' for the evaluation of FLM but has the disadvantage to require a certain technical expertise and to be time consuming. Level of PG, the predominant anionic PL in human neonates, start to rise after 35 weeks of gestational age.[44,86,114] PG levels may be determined by a rapid agglutination test and are not influenced by sample contamination. Surfactant to albumin ratio has become a widely used rapid automated test to evaluate FLM. Lamellar body counts on amniotic fluid are another test frequently used for FLM testing.[44,86,114] A number of other tests exist for the assessment of FLM and are reviewed elsewhere.[44] Any of the tests used for FLM indicating a mature result have a strong predictive value for the absence of NRDS.[44,86,114] It has therefore been suggested to use a sequential approach to evaluate FLM. For example, a rapid inexpensive test is performed first (i.e. surfactant/albumin ratio or lamellar body count) and if its result clearly indicates lung maturity or immaturity, no further testing is required. However, intermediate results make further testing necessary.

Surfactant replacement therapy

Successful surfactant therapy for NRDS was first demonstrated by Fujiwara *et al* in 1980[115] and in the following years this treatment significantly reduced mortality in premature infants. Since 1989 surfactant therapy has been part of the standard of care for neonatal infants with NRDS requiring mechanical ventilation.[27,116] Two different choices are available for surfactant replacement therapy: native surfactant and synthetic surfactant.[27] Native surfactants are lung surfactant extracts from bovine or porcine sources. These products contain varying levels of the hydrophobic surfactant proteins SP-B and SP-C. Compared to synthetic preparations, natural surfactants have better biophysical activity and resistance to inactivation by serum proteins and inflammatory mediators.[117,118] Synthetic lung surfactants offer the advantage of having a more uniform composition and no potential risk for disease transmission (i.e. bovine spongiform encephalopathy) or allergic sensitization to surfactant proteins.[27] In recent years, synthetic surfactants have been developed that contain recombinant or analog surfactant proteins.[27] Multiple methods have been used to administer exogenous lung surfactants. Nebulization is difficult because the thickness of surfactant prevents efficient delivery to the lower airways. Direct tracheal or bronchial instillation is most often utilized. Patients need to be

repositioned during administration to allow a more even distribution of surfactant within the lung.[119]

For the treatment of NRDS, surfactant can be given as prophylactic therapy or rescue therapy.[27,85] Natural surfactants are the treatment most preferred by neonatologists.[85] In general, the earlier surfactant is administered in the course of NRDS the better are the chances for improved clinical outcome, through the prevention of lung inflammation and injury. Prophylactic therapy (within 15 minutes of birth) is recommended for all infants under 27 weeks of gestation or for infants under 30 weeks of gestation if they require intubation or if the mother has not received prenatal corticosteroids.[85] The 'INSURE' technique (intubate surfactant extubate to CPAP) used for prophylactical surfactant therapy has been shown to reduce the need for mechanical ventilation in premature infants.[85] Early rescue therapy is indicated in untreated infants that show evidence of NRDS such as an increasing requirement for oxygen or a need for mechanical ventilation.[85] Each dose of surfactant typically consists of a minimum of 100 mg of phospholipid (PL) per kg body weight.[85] A second or third dose of surfactant are required if there is ongoing evidence of NRDS.

Surfactant studies in large animals

Lung surfactant composition and function have been described in several large animal species. In particular, bovine and porcine surfactant characteristics have been thoroughly described since these surfactants are available commercially for the treatment of human NRDS.[120-122] Surfactant alterations have been reported in neonates and adolescent animals of several large animal species.[38,40,41,123] Large animals have been used as models for the development of ARDS and ALI induced by different techniques including repeated lung lavages, inhalation of toxic gases, and induction of sepsis or endotoxemia.[124-126] However, only limited data exists on surfactant alterations associated with naturally occurring respiratory disease in adult large animal species. Most data is available from the horse in which BALF analysis is routinely performed as a diagnostic procedure.[21-24,26]

Cattle

RDS due to surfactant deficiency affects premature calves delivered naturally or by caesarian section.[127,128] Lung surfactant maturation occurs relatively late in the bovine species compared to humans and sheep.[127] Calves born close to 270 days of gestational age (average gestational length 278 days) are still considered at risk for the development of RDS.[127-129] Calves of the Belgian White Blue (BWB) breed appear more susceptible to develop RDS than other breeds. Surfactant from newborn BWB calves with RDS is characterized by a significantly lower SP-C content, an increased surface tension, and a modified PL profile when compared to healthy control calves of the same breed.[41] Surfactant from healthy BWB calves, at several months of age, contains significantly lower levels of SP-B, SP-C, and PC compared to surfactant from healthy Holstein Friesian calves.[40] These findings suggest that surfactant alteration in BWB calves may predispose this breed to the development of RDS and other respiratory diseases.

FLM testing is applicable to the bovine species.[128,130] Similar to findings in human medicine, amniotic fluid L/S ratios above 2 are indicative of lung maturity in the neonatal calf.[128] L/S ratios determined from amniotic fluid of 35 calves delivered via caesarian section close to term, showed significantly lower L/S ratios (L/S =1.5) for calves affected with RDS than for calves that did not develop the disease (L/S = 2.6).[128] Furthermore, lung surfactant maturation in the bovine fetus is enhanced by administration of corticosteroids or prostaglandins.[130] Cows between 258 and 270 days of gestation (n = 45) were treated with a corticosteroid (flumethasone) or a prostaglandin $F_{2\alpha}$ (dinoprost), two treatments which are used to induce parturition. L/S ratios were determined before treatment and 30 hours later. L/S ratios were significantly higher in cows treated with either corticosteroids or prostaglandins, both compared to pretreatment values and cows treated with saline.[130] Calves delivered from treated cows were less acidotic than those from control cows. Both treatments were therefore considered to promote pulmonary maturation equally. Even though FLM testing is not performed as a routine procedure in bovine medicine, it may be of benefit for the assessment of fetal well-being in pregnancies that are the product of assisted reproductive techniques.[131]

Sheep

Similar to calves, preterm lambs born before 130 days of gestation (gestational length = 148 days) suffer from RDS due to surfactant deficiency.[132,133] The preterm lamb has been used extensively as an animal model to study the development, prevention, and treatment of RDS.[134-136] Details on these studies are available in the literature.[134,137-140] As in other animal species, lung surfactant composition undergoes significant changes towards the end of gestation. A significant rise in PC is described in fetal sheep at 3-4 days before birth.[137] In contrast to the human newborn, PI rather than PG is the predominant anionic PL in term lambs.[137] Expression of both SP-A and SP-D increases towards the end of gestation.[141] Expression of SP-A and SP-D in lambs is also influenced by the type of pathogen present in the lung.[142] For example, infection of neonatal lambs with parainfluenza 3 (PI-3) virus induces enhanced expression of SP-A and SP-D in parallel with decreased viral replication.[143] Conversely, infection of 3-months old lambs with *Mannheimia hemolytica* results in decreased SP-D expression.[144]

Pigs

Fatal RDS, also called 'barker syndrome', occurs with increased frequency in neonatal large white pigs.[145,146] Affected animals are characterized by lung immaturity with hyaline membrane formation, lung surfactant deficiency, and abnormally small thyroids with corresponding clinical signs of hypothyroidism.[146] The disease is likely transmitted as an autosomal recessive trait.[146,147]

Surfactant composition in healthy newborn piglets differs from that of adolescent animals.[38] Neonatal pig surfactant is characterized by higher levels of PI (predominant anionic PL in neonatal pigs), higher levels of SP-B and SP-C, and a higher proportion of unsaturated PC molecular species compared to surfactant from adolescent pigs. These compositional changes are believed to be responsible for an enhanced ability to lower surface tension found in neonatal pig surfactant.[38]

Horses

The role of surfactant deficiency in equine RDS has been the subject of debate. At a recent meeting by the Dorothy Russell Havemeyer Working Group on ALI and ARDS in

veterinary medicine, the term neonatal equine respiratory distress syndrome (NERDS) was described as a separate syndrome from equine acute neonatal respiratory distress syndrome (EqNARDS).[148] NERDS was defined as a primary surfactant deficiency due to failure of final fetal pulmonary surfactant metabolism maturation. A number of criteria were defined for the diagnosis of NERDS. These criteria include: (1) persistent hypoxemia and progressive hypercapnia, (2) presence of risk factors (i.e. gestational length <290 days or <88% of average dams previous gestational lengths, induction of parturition, or C-section), (3) failure to develop normal immediate postpartum respiratory pattern, (4) ground glass appearance of lateral radiographs at less than 24 hours of age, (5) absence of fetal inflammatory response syndrome, and (6) absence of congenital cardiac disease.

FLM testing is not considered a reliable test in equine neonatal medicine.[35,149,150] L/S ratios and PG levels measured from amniotic fluid (collected either by transabdominal amniocentesis, at parturition or during C-section) failed to predict lung maturity in several studies. In addition, amniocentesis is associated with a higher risk of abortion, placental separation and possible infection when compared to human medicine.[35,150,151] Recently, the determination of lamellar body counts from amniotic fluid collected during delivery of term foals was evaluated.[152,153] The usefulness of this test to evaluate lung maturity of foals born prematurely remains to be determined. Prevention of NERDS in the foal is limited by the fact that the equine placenta is relatively impermeable to exogenous corticosteroids.[36] Betamethasone given intramuscularly to the equine fetus under ultrasound guidance is known to hasten lung maturation but has been associated with premature delivery.[154]

Administration of exogenous surfactant to premature foals at risk for RDS has been attempted in a small number of animals but has been of variable success and is limited by the high cost of this treatment in large animals.[36,155,156] Surfactant in foals can be administered by a long catheter through a nasotracheal tube. The foal should be repositioned several times (left lateral, right lateral, sternal and dorsal recumbency) and ventilated with a resuscitation bag for 5 to 10 minutes to distribute surfactant into the distal airways.[155,157] Surfactant therapy may also be useful in foals suffering from meconium aspiration syndrome.[158]

Our group recently reported that lung surfactant in neonatal foals is significantly different from that in adult horses.[123] Surfactant from neonatal foals at less than 24 hours of age had higher surface tension, higher levels of PI, and lower levels of PG compared to adult horses. Compositional differences in foal surfactant potentially influence both biophysical and immunomodulatory functions of surfactant.

Surfactant proteins SP-A and SP-D have been purified and characterized in healthy adult horses.[159-161] Extrapulmonary locations of SP-A and SP-D thus far described in the equine veterinary literature include: joints and both mare and stallion reproductive tracts.[162-164] Surfactant alterations occur in horses that undergo prolonged transportation, anesthesia, or intense exercise.[21-23] Transport of horses for a prolonged period of time results in a decrease in surfactant PG concentration and decreased levels of SP-A and SP-D compared to pre-transport levels.[21,26] Anesthesia of horses (maintained in lateral recumbency) is associated with a decreased amount of surfactant recovered from the dependent versus independent lung at 72 after anesthesia.[22] Mechanisms and consequences of these surfactant alterations remain unclear. Surfactant from horses undergoing exercise with an intensity sufficient to induce intrapulmonary hemorrhage demonstrated increased surface tension in the absence of compositional changes.[23] These results may be explained by accumulation of blood or hemoglobin in the alveoli of affected horses which are known as inhibitors of surfactant function.

Surfactant from RAO-affected horses is characterized by decreased levels of PG at advanced disease stages.[24] Preliminary data suggest that surfactant alterations in horses clinically affected by RAO resemble those described in asthmatic patients and include an altered surfactant aggregate ratio (decreased LA/SA), decreased levels of PG, and a tendency for increased surface tension.[25] It remains to be determined if surfactant alterations in RAO-affected horses are related to or influence disease severity.

Treatment with the surfactant-rich fraction of BALF, isolated from a freshly euthanized horse, has been reported in one gelding suffering from near-drowning syndrome and appeared to contribute to clinical improvement.[165]

Conclusion

The role of surfactant in human NRDS is well established as is emphasized by the use of FLM testing in high-risk pregnancies and treatment with exogenous surfactant of premature infants at risk or with NRDS. The influence of surfactant alterations on other respiratory diseases such ARDS or asthma warrants further investigation. Similarly, treatment with exogenous surfactant in these diseases is still under scrutiny and new surfactant formulations need to be evaluated. Even though the importance of surfactant deficiency is recognized in several large animal neonates, treatment with exogenous surfactant is largely cost-prohibitive. Treatment with exogenous surfactant has been used in the neonatal foal with variable success. Due to cost limitations of treatment, there are no studies that have evaluated this treatment on a larger number of animals and under standardized conditions. FLM testing in large animal medicine may be applied in highly valued cattle. A reliable test used to predict FLM in the equine fetus is desirable but not yet available. Recently there has been an interest in evaluating SP-A and SP-D in sheep in relation with respiratory infections. Although typically consistent with reports in humans, overall information on surfactant alterations in veterinary medicine remains limited.

Table I.1.1.: Lung surfactant composition.

Phospholipids 80-85%		
Phosphatidylcholine	PC	75% (50% DPPC)
Phosphatidylglycerol	PG	12%
Phosphatidylethanolamine	PE	5%
Phosphatidyinositol	PI	4%
Phosphatidyserine	PS	2%
Sphingomyelin	SPM	1.5%
Lysophospholipid	LPL	<1%
Proteins 5-10%		
SP-A	50-70%	
SP-B	10%	
SP-C	10%	
SP-D	10-30%	
Neutral Lipids 5-10%		
Cholesterol	90-95%	

Table I.1.2.: Mechanisms of lung surfactant alteration

Surfactant deficiency: insufficient synthesis/storage/release
Immaturity type II alveolar cells
Injury type II alveolar cells
Surfactant inhibition: accumulation of surfactant inhibitors
Plasma proteins
Hemoglobin
Cellular lipids
Meconium
Surfactant degradation: by inflammatory enzymes or products
Proteases
Phospholipases
Reactive oxygen species

Figure I.1.1.: Molecular structure of phospholipids. Phospholipids are amphipathic structures with a polar (hydrophilic) and non-polar (hydrophobic) region. Phospholipid class is determined by the polar head group X and can be further subdivided into different molecular species based on length and degree of saturation of the fatty acid chains (FA_1 and FA_2). Polar head group and non-polar fatty acid chains are attached to a 3-carbon glycerol backbone.

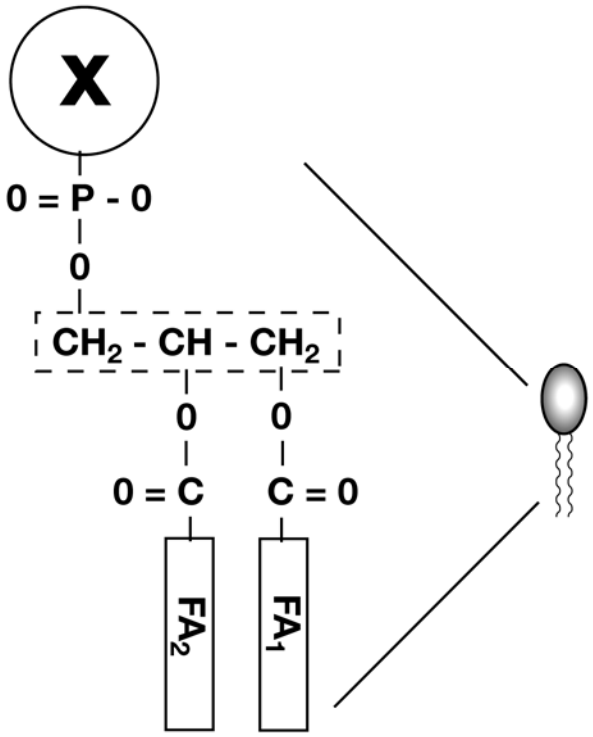


Figure I.1.2.: Levels of PC, PG, PI, and SPH in human amniotic fluid during gestation (modified from Cunningham FG, MacDonald PC, Gant NF, Leveno KJ, Gilshap LC, editors. Williams Obstetrics, 19th ed. Appleton and Lange. 1993:188. Copyright: The McGraw Companies, Inc.).

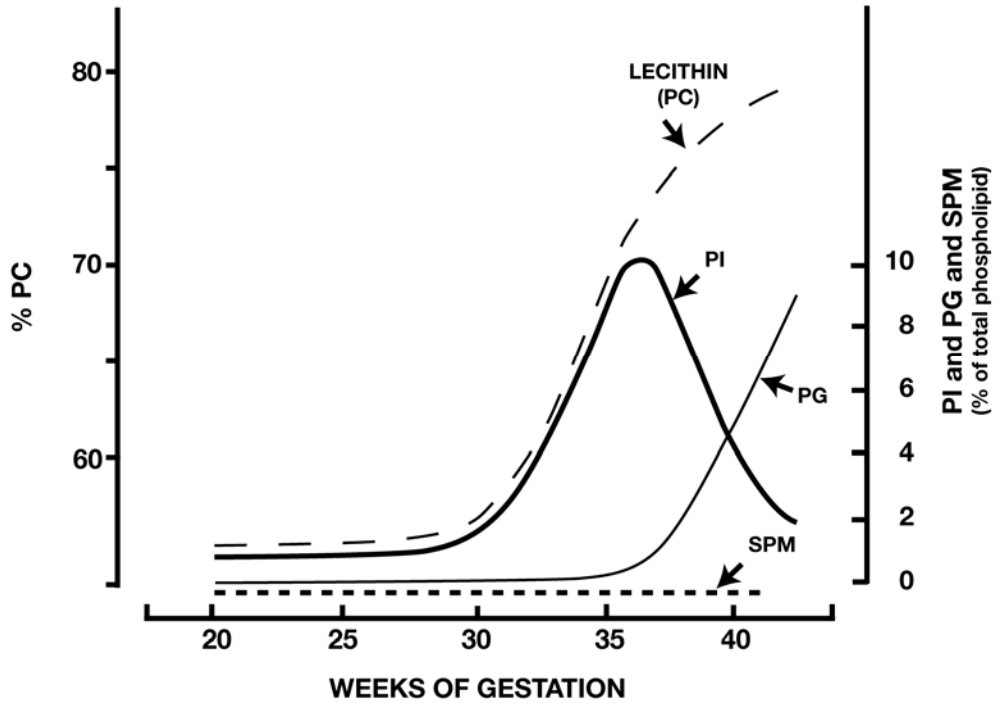


Figure I.1.3.: Structure of surfactant proteins (SP-A, SP-B, SP-C, and SP-D).

(A) SP-A and SP-D are hydrophilic surfactant proteins and part of the collectin family. Common structural features are an amino N-terminal, a collagen like domain, a neck region, and a carbohydrate recognition domain (CRD).

(B) SP-B and SP-C are hydrophobic surfactant proteins and play a role in biophysical surfactant functions. They are found in close association with surfactant phospholipids.

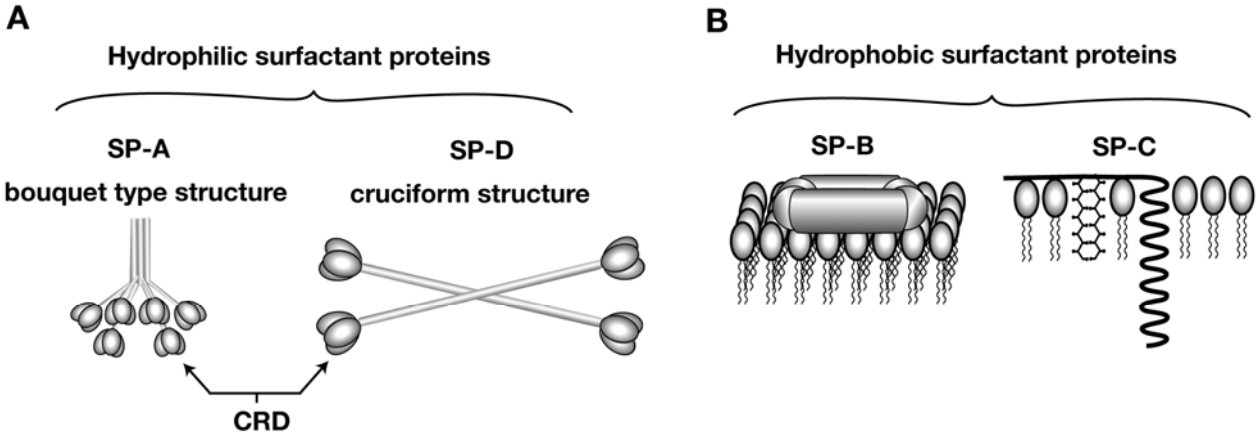
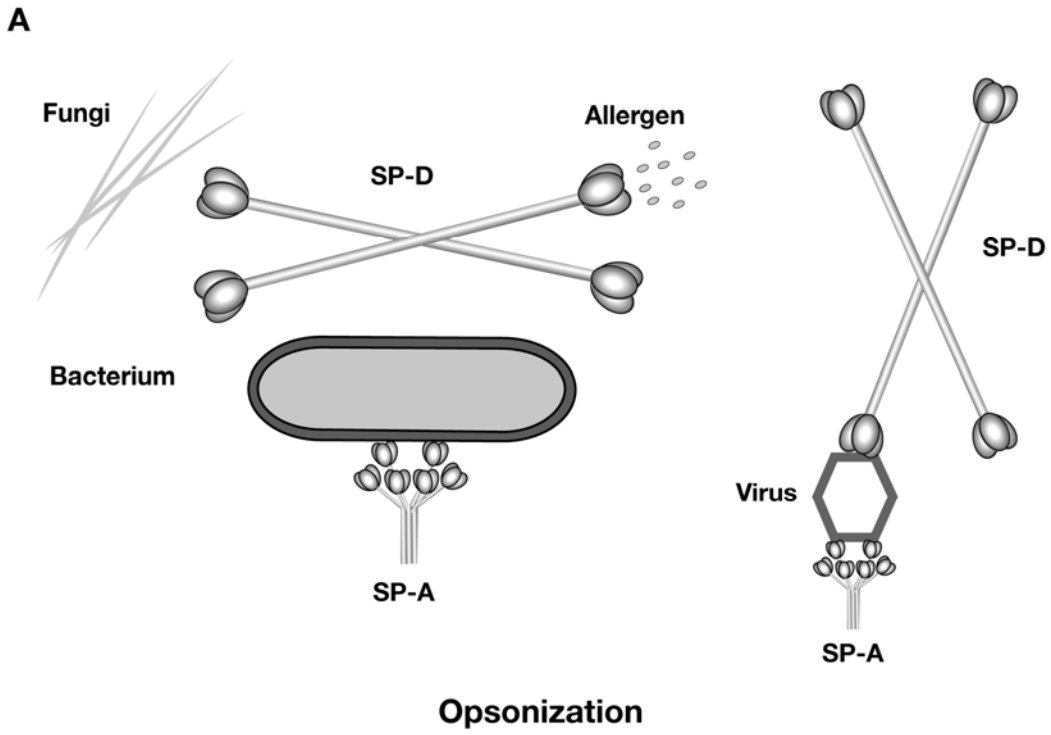


Figure I.1.4.: Schematic representation of surfactant host defense functions, mainly assured by SP-A and SP-D.

(A) Opsonization of bacteria, viruses, fungi, and allergens.

(B) Enhanced phagocytosis of pathogens, increased phagocytosis of apoptotic cells, and regulation of inflammatory mediator production.



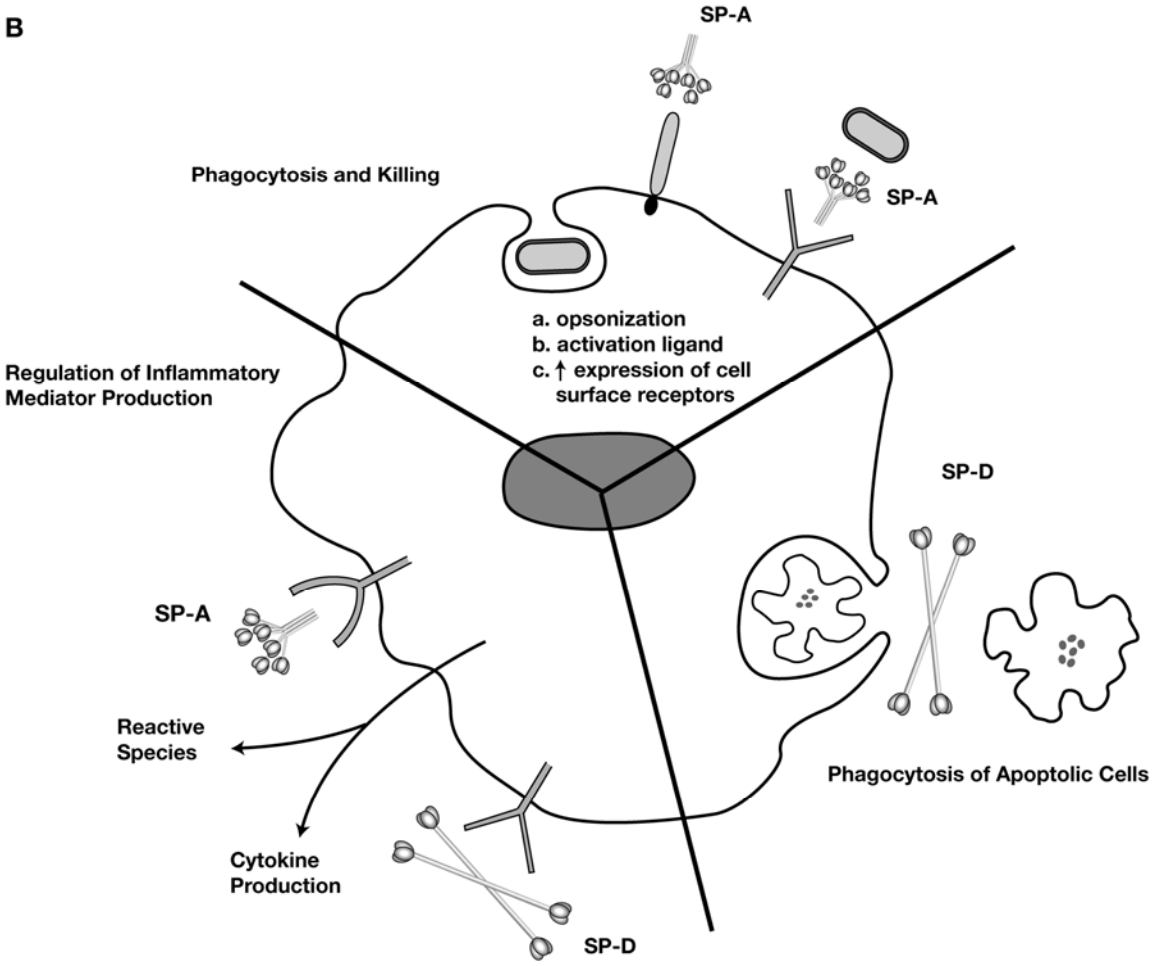
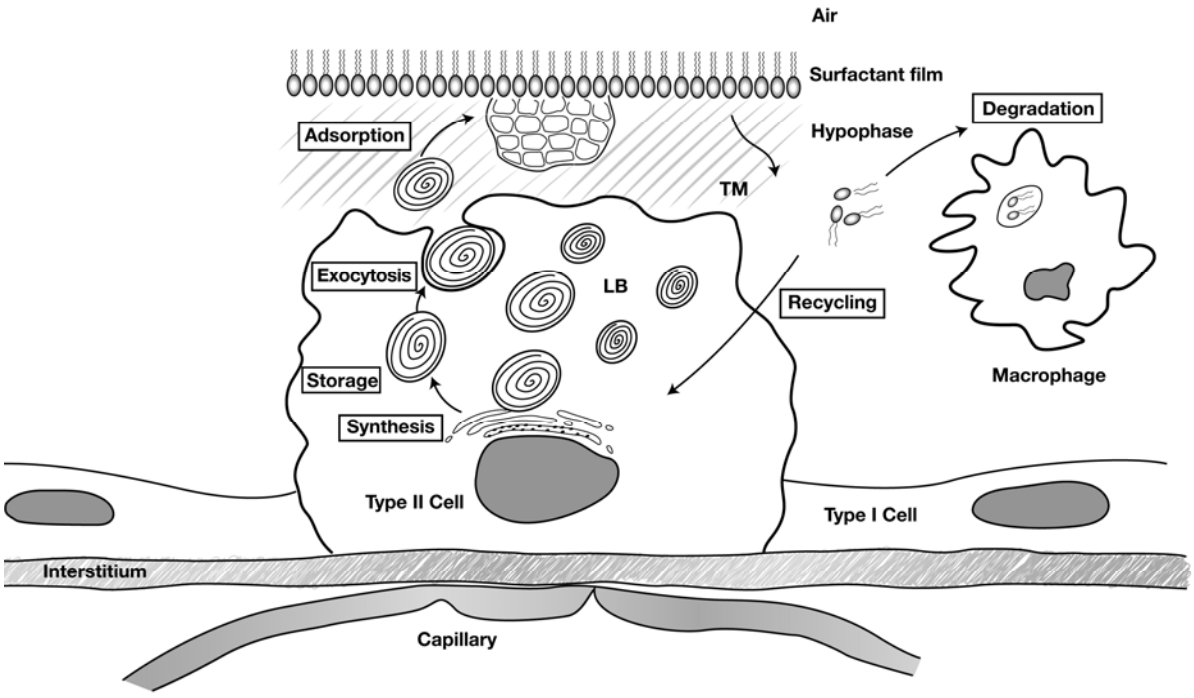

B
SP-A
Phagocytosis and Killing
SP-A
a. opsonization
b. activation ligand
c. ↑ expression of cell surface receptors
Regulation of Inflammatory Mediator Production
SP-D
SP-A
Reactive Species
Phagocytosis of Apoptolic Cells
Cytokine Production
SP-D

Figure I.1.5.: Schematic representation of surfactant metabolism: surfactant is synthesized by type II alveolar cells. Surfactant is stored in specialized structures called lamellar bodies (LB) and exocytosed to the alveolar hypophase, where it exists in form of heterogenous phospholipid-rich aggregates including tubular myelin (TM). Surfactant components adsorb to the air-liquid interface where they actively reduce surface tension. A large part of surfactant is recycled by type II alveolar cells whereas a small part is degraded by alveolar macrophages.



Section 2:
Role of Surfactant in Asthma and Related Models

Asthma is a disease characterized by reversible airway obstruction, mucus accumulation, bronchial hyperresposiveness, and airway inflammation.[104] The pathophysiology of asthma may be influenced by alterations in airway surfactant. The potential role of surfactant in airway disease was described over 30 years ago.[53] Since that time airway surfactant has been attributed a number of functions that can potentially influence the severity of airway disease. These functions include: maintain airway patency, improve muco-ciliary clearance, reduce severity of bronchoconstriction, and downregulate allergic airway inflammation. This article is meant to give an overview of airway surfactant and its role in asthma.

Airway surfactant origin, composition and function

Airway surfactant originates from alveolar surfactant.[43] A fraction of surfactant produced in the alveoli leaks to the airways and spreads along the smaller airways by a combination of pressure differences and muco-ciliary clearance.[166,167] Evidence for the existence of airway surfactant was provided by visualization of a surfactant film lining the airways via electron microscopy,[168] and isolation of surfactant components from tracheal wash[43] and sputum.[105] The trachea is able to synthesize phospholipids locally. These phospholipids have a very different composition than those found in alveolar surfactant (containing mostly unsaturated PC species) and are retained in the tracheal mucosa (rather than secreted into a surfactant film).[169] Airway epithelial cells and Clara cells are unable to synthesize DPPC which is a key component of functional surfactant.[166] In addition, lamellar bodies, the principal storage form of surfactant in the alveolus, have not been identified in any of the cells lining the airways. Clara cells and submucosal cells in the airways are able to synthesize surfactant specific proteins SP-A, SP-B, and SP-D.[8] SP-C is exclusively synthesized in the alveoli.

Direct analysis of airway surfactant is very challenging since it is virtually impossible to selectively sample airway surfactant. A separation of BALF into aliquots collected early versus those collected late may allow obtaining samples that are somewhat representative of small airway versus alveolar surfactant. However, this technique as well as tracheal lavage fluid collection will always contain a percentage of alveolar derived surfactant. An extensive analysis

of surfactant was performed at different levels of the lung and respiratory tree of the pig.[43] Phospholipid composition in tracheal wash of pigs closely resembled that of BALF but was distinctively different from phospholipid composition of the underlying epithelium and lung parenchyma.[43] Tracheal lavage fluid contained reduced levels of SP-A and almost no SP-B and SP-C.[43] In contrast to alveolar derived surfactant, phospholipid composition of airway epithelium was characterized by lower levels PC and PG, higher levels of PE, and a higher proportion of unsaturated PC molecular species.[43,169]

Function

Surface tension in the conducting airways is higher than in the alveoli (<2mN/m) and approaches values of 30 mN/m.[43] Airway surfactant has been attributed a number of physiological functions which are briefly explained in the next paragraphs.

Airway patency

Surfactant helps maintain patency of small collapsible airways by its ability to lower surface tension. Surfactant prevents 'compliant collapse' as illustrated by the law of Laplace for a cylinder $P=\gamma/r$. Small compliant airways have a higher tendency to collapse if surface tension is high.[170,171]

Edema prevention

Surfactant exerts hydrostatic forces within the airways that act against fluid influx into the airways and edema formation.[5] Furthermore, airway surfactant protects the airways against desiccation as part of the epithelial lining fluid.[172] The latter mechanism was demonstrated in a series of *in vitro* and *in situ* experiments.[54,55,173]

Muco-ciliary clearance

Airway surfactant is located between the sol and gel phases of mucus and at the lining of the mucous surface.[168] The sol phase is a liquid layer that overlays airway epithelial cells whereas the gel phase is more viscous and in contact with the air. Surfactant is believed to allow sliding of the gel phase over the sol phase. Surfactant improves muco-ciliary clearance by regulating the proportion of gel and sol phase, reducing mucus adhesiveness, and increasing

ciliary beat frequency.[4,56] Surfactant promotes movement of inhaled particles or pathogens from peripheral to more central airways.[174]

Barrier function

Surfactant forms a non-specific barrier against the inhalation of pathogens. It has been suggested that surfactant masks bronchial irritant receptors and reduces severity of bronchoconstriction in response to inhaled allergens.[175] This theory was enforced by the fact that aerosolized surfactant inhibits acetylcholine bronchoconstriction in rats[172] and that surfactant depletion leads to exacerbated bronchoconstriction following administration of certain drugs or allergens.[170]

Immunomodulation

Surfactant proteins SP-A and SP-D play an important role in innate immunity and immune-modulation of the lung. SP-A and SP-D influence allergic airway inflammation at different levels.

SP-A and SP-D are contained in the airway lining fluid and are therefore directly exposed to inhaled allergens. SP-A and SP-D are able to opsonize a variety of allergens (i.e. pollen grains, mite allergens, aspergillus fumigates allergens).[176-179] They also inhibit allergen induced histamine release and Ig-E dependent mast cell degranulation. SP-A and SP-D modulate activation of alveolar macrophages and dendritic cells and inhibit T-cell differentiation during the acute stage of the allergic response. These mechanisms tune the inflammatory response towards a Th1 cytokine profile rather than Th2 cytokine profile. Finally, these surfactant proteins bind to surface bound DNA and enhance the removal of apoptotic cells. It appears that SP-A and SP-D modulate pulmonary immune response: inflammatory response is suppressed in healthy, unchallenged lungs whereas it is upregulated in lungs exposed to pathogens or aeroallergens.[65]

A number of studies have evaluated the role of SP-A and SP-D in allergic airway inflammation and asthma. The present review is focused on the role of surfactant phospholipid rather than surfactant protein.

Mechanisms of surfactant alteration

Increased surface tension of surfactant can be caused by surfactant deficiency (quantitative deficit of surfactant) or surfactant dysfunction (qualitative change in surfactant).[3] Surfactant deficiency may be the result of an immaturity or injury to type II alveolar cells leading to insufficient surfactant synthesis, storage, or release. Surfactant dysfunction or loss of surface activity can be caused by a variety of surfactant inhibitors or by the degradation of surfactant components.[3]

Surfactant inhibitors include plasma proteins (i.e. albumin, fibrinogen or fibrin), hemoglobin, cellular lipids (i.e. non-saturated phospholipids, lysophospholipids, FA, cholesterol), and meconium.[180] Inhibition of surfactant function occurs via two main mechanisms: competitive adsorption and film penetration/fluidization.[180] Competitive adsorption is caused by plasma proteins which also have an amphipathic structure and therefore possess an intrinsic surface activity.[180] These large proteins compete with phospholipids for adsorption to the air-liquid interface. Film penetration/fluidization results from introduction of lysophospholipids, FA, or other membrane lipids into the surfactant film.[180] These lipids have the greatest effect on surface activity during compression.

Degradation of surfactant components occurs via the action of proteases, phospholipases or reactive oxygen species liberated during pulmonary inflammation. Neutrophil elastase is one example of an inflammatory protease that has been shown to alter surface activity of surfactant by inactivating surfactant proteins.[82] Phospholipases hydrolyze phospholipids and produce lysophospholipids and FA that interfere with surfactant film fluidity.[83,84] Decreased levels of SP-B, altered surfactant aggregate formation and metabolism are additional mechanisms that have been proposed for surfactant dysfunction.[3]

In vitro and *in situ* models of surfactant function in airway disease

The following paragraphs will discuss methods used to evaluate surfactant function under conditions that mimic those found in the smaller airways. Methods used to evaluate surfactant behavior in the alveoli are discussed elsewhere.

Several *in vitro* and *in situ* methods have shown that surfactant is superior to saline in its ability to maintain patency of small capillaries. These models allowed the investigation of the action of different inhibiting agents on surfactant function.[54,55,81,173,181-183]

In vitro models

Capillary surfactometer

The capillary surfactometer is an *in vitro* model used to mimic and study the behavior of different liquids in the airway.[54] It contains a glass capillary with a small narrowed area (see Figure I.3.4.).[181] This section of the capillary has an inner diameter of 0.24 to 0.25 mm which is the approximate width of a human terminal airway.[184] A constant airflow is maintained through the capillary. If liquid is introduced it accumulates in the narrow section of the capillary, pressure within the capillary rises until the liquid is pushed out and pressure drops back to zero. If the liquid accumulation reforms, the pressure rises again. The capillary surfactometer measures the percentage of time that the capillary remains open to airflow over 2 minutes.

When surfactant is introduced into the narrowed capillary of the capillary surfactometer, it does not form a liquid plug but keeps the capillary open to airflow.[54] Conversely, saline or solvent re-accumulates in the capillary and reduces the percentage of time it remains open.[54] The ability of pulmonary surfactant to maintain airflow through the narrowed capillary is lost if surfactant is very dilute or contains inhibiting proteins (i.e. albumin and fibrinogen).[54] These experiments were repeated replacing the glass capillary *in situ* by a rat esophagus with a narrowed section.[54] The latter allowed to demonstrate that surfactant adopted the same behavior in a soft compliant tube (rat esophagus) than in a stiff glass capillary.

In situ models

Following the previously mentioned experiments using capillary glass tubes, terminal conducting airways from rats and piglets were used to study the effect of surfactant on smaller airway patency.[55,173,183] These models consisted of using excised lungs and delivering airflow to a small peripheral airway and exiting through the trachea. The pressure of air entering the airway was measured. If the airway maintained patency, the pressure remained low whereas if a blocking liquid column formed, the pressure increased rapidly and dropped abruptly when the

blockage was alleviated. Rat airways flushed with saline only remained open briefly whereas flush with CLSE resulted in a prolonged period of openness.[55] Removal of endogenous surfactant resulted in blockage of airways while administration of aerosolized surfactant increased airway patency.[183] In piglet airways patency was decreased in surfactant deficient conditions but was restored with administration of exogenous surfactant.[173]

Evidence for surfactant inhibition and/or degradation

Several mechanisms of surfactant inhibition and degradation were demonstrated with the methods described in the previous sections.

Surfactant inhibition may develop following protein infiltration into the alveoli and airways following inflammation.[15,182] Experiments using the capillary surfactometer have demonstrated the effect of albumin and fibrinogen on surfactant function. Albumin (at a concentration above 10mg/ml) and fibrinogen (at a concentration of 0.5mg/ml) added to surfactant introduced into the capillary surfactometer were shown to disrupt the ability of surfactant to maintain the capillary open. Lower concentrations of albumin in surfactant induced the same effect if samples were assessed at a temperature below 25ºC induced similar results.

Surfactant degradation may occur by the action of different enzymes. Secretory phospholipase A 2 ($sPLA_2$), is an enzyme able to hydrolyze surfactant phospholipids. The effect of $sPLA_2$ on surfactant function has been investigated by several groups of researchers.[83,106,185-188] Different classes of $sPLA_2$ exist and are secreted by a variety of inflammatory or airway cells. Depending on the type of $sPLA_2$ present, hydrolysis preferentially affects a specific class of phospholipid.[106] Phospholipid hydrolysis results in production of lysophospholipids, FA, and a decrease in phospholipid content.[187] Lysophospholipids were shown to have the strongest *in vitro* ability to disrupt surfactant function.[185,187] Fatty acid production also decreased surfactant function, but to a lesser extent. In comparison, an 80% depletion in phospholipid content was necessary to induce surfactant dysfunction.[187] Addition of phospholipase C, but not $sPLA_2$, seriously disrupted surface activity evaluated with the capillary surfactometer in one study.[81] Recently, it was reported that the combined action of eosinophil sPLA2 and lysophospholipase exacerbates surfactant dysfunction.[83,188]

In vitro experiments demonstrated that cooling exacerbated protein inhibition of CLSE, of surfactant isolated from mice infected with RSV, and of surfactant isolated from allergen challenged asthmatic patients.[182]

Laboratory animal models of surfactant phospholipid alterations in allergic airway disease

Guinea-pig model of surfactant alteration in asthma

The guinea-pig is a popular model of allergic airway disease and has been used to study surfactant abnormalities.[13,14,189-194] Guinea-pigs are easily sensitized to antigens and develop acute bronchoconstriction with pulmonary eosinophilia following antigen challenge.[190] In contrast to human asthma, the allergic response in guinea pigs can be induced by a variety of antigens, is mediated by a subclass of IgG (rather than IgE), and the correlation between lung eosinophilia and airway hyperreactivity is poor.[190] Even though the guinea-pig is most often used as an acute model of asthma, it is also possible to elicit a more chronic response (see Table I.2.1.)..[13,14,189-194]

Acute challenge models

Guinea-pigs actively immunized against ovalbumin (ovalbumin injection) develop significant changes in lung function (decreased tidal volume, minute volume, dynamic compliance and increased airway resistance) [13,193] and arterial blood gas[193] when subsequently challenged with aerosolized ovalbumin. Similarly, ovalbumin sensitized guinea-pigs when challenged developed protein infiltration into airways that lead to surfactant inhibition.[13] Surfactant from 'immunized-challenged' guinea-pigs demonstrated increased surface tension and total protein content but unchanged phospholipid content and composition when compared to immunized non-challenged animals.[13]

Prophylactic administration of surfactant reduces the severity of airway obstruction in immunized challenged guinea pigs.[193] Treatment of immunized challenged animals with surfactant (tracheal instillation of CLSE) decreased changes in lung function and arterial blood gas typically seen following allergen challenge.[193]

Indirect evidence suggests that surfactant degradation contributes to loss of surface activity in the guinea-pig model of acute asthma.[191] Surface tension was increased in BALF from immunized challenged animals versus non-immunized challenged animals following 4 hour incubation at 37ºC.[191] No change in surface tension was seen if the incubation was performed at 4ºC.[191] Surface tension increased if surfactant pellets were incubated (4h, 37ºC) with surfactant supernatant but did not increase with saline incubation.[191] The increase in surface tension induced by incubation was partially suppressed by addition of a phospholipase A2 inhibitor or protease inhibitors.[191] These experiments indicated the possible presence of enzymes capable of surfactant degradation.

Repetitive challenge models

Repeated exposure to allergen induces reduced surfactant synthesis, decreased surface activity, and altered phospholipid composition.[14,194] BALF from immunized guinea-pigs repetitively challenged with ovalbumin demonstrated a decreased surface activity and increased protein content compared to non-immunized challenged animals.[14] Type II alveolar cells from animals immunized and then challenged with ovalbumin had a lower phospholipid content and decreased phospholipid synthesis (as shown by their ability to incorporate a ^{3}H labeled surfactant precursor).[14] Another study evaluated guinea-pigs sensitized to ovalbumin by inhalation (nebulized every day for 10 minutes over 10 days).[194] This model resulted in increased BALF cell counts and total protein in guinea-pigs sensitized with ovalbumin versus saline.[194] Surfactant from these animals was characterized by an increased surface tension, decreased phospholipid content in large aggregates, and altered phospholipid composition (decreased PC and PG, increased PI).[194]

Human asthmatic patients

Surfactant has been evaluated in asthmatic patients following allergen challenge,[16-18,20] and in asthmatic patients with clinical disease (see Table I.2.2.).[19,105]

Allergen challenge

Bronchoscopy offers the possibility to perform an allergen challenge in a specific lung area and to use a saline challenge on the opposite side. This technique allows using the patient as

its own control and also minimizes adverse clinical effects of allergen challenge. In brief, asthmatic patients with mild, well controlled disease are used. Patients are tested via a skin prick test to identify the allergen to which they are sensitive. Allergen dose to be used in the challenge is determined either by inhalation of increasing doses of allergen or by evaluation of skin prick reaction to different doses of allergen. By bronchoscopy patients are challenged with allergen on one side and saline on the contralateral side of the lung.

Several studies evaluated surfactant alterations in asthmatic patients following allergen challenge. Allergen challenge induced an acute inflammatory reaction of the lung characterized by infiltration of inflammatory cells and protein. Surfactant dysfunction developed following allergen challenge in the majority of studies.[16-18,20] Several mechanisms have been proposed as causes for surfactant dysfunction induced by allergen challenge of asthmatic patients and include: surfactant inhibition by non-specific protein infiltration,[16,17,20] surfactant inhibition by specific proteins,[107] and surfactant degradation by $sPLA_2$.[18]

Protein infiltration into the airways is believed to be one of the major mechanisms leading to surfactant dysfunction. This mechanism was proposed based on findings from studies on *in vitro* models[81], animal models of asthma[13,191], and asthmatic patients.[16,17,20] *In vitro* models have demonstrated the inhibiting effect of proteins on surfactant function.[81] Additional evidence for protein inhibition of surfactant in allergen challenged asthmatic patients was provided by the fact that washing of surfactant pellets with saline restored their ability to lower surface tension and that incubation of CLSE with surfactant supernatant but not saline resulted in dysfunctional surfactant.[16,17] Analysis of BALF phospholipid composition revealed an increase in PC content in allergen challenged asthmatics.[20] A detailed analysis of PC molecular species showed a decrease in DPPC related to an increase of PC species containing linoleic acid. Since these PC species are characteristic of plasma (where they are protein-bound), it was speculated that lipoprotein infiltration of the airways caused the alterations in phospholipid composition. This speculation was supported by a strong correlation between one of the PC molecular species (16:0/18:2) and protein content. Finally, correlations existing between protein content of BALF and surface tension in different studies also provide evidence for a major role of proteins in surfactant dysfunction.[16,17,20]

Surfactant inhibition by a specific protein has also been suggested. Eosinophils are important effector cells in the asthmatic response. Eosinophil cationic proteins (ECP) are released by activated eosinophils. Levels of ECP are elevated in asthmatic patients following allergen challenge.[107] ECP levels were shown to correlate with surfactant dysfunction. *In vitro* experiments demonstrated a concentration dependent inhibition of surfactant function that was reversed by ECP antibodies. ECP was more potent in causing surfactant dysfunction and ultrastructural surfactant changes than albumin or fibrinogen.

Hydrolysis of surfactant phospholipids has been proposed as an additional mechanism leading to surfactant dysfunction. Allergen challenge in asthmatic patients was shown to induce a significant increase in $sPLA_2$ and arachidonic acid 4 hours following challenge.[195] One study that evaluated surfactant alterations in response to allergen challenge found that surface tension evaluated from washed pellets was dysfunctional in some of the asthmatic patients (responders) but not in others (non-responders).[18] Data analyzed from these subsets of patients revealed that responders had significantly lower anionic phospholipid content (PG and PI) compared to non-responders. Correlations between phospholipid variables (LA/SA, PG, and PC/PG) and surface tension were stronger than those between protein and surface tension. In addition, BALF from allergen challenged asthmatic patients demonstrated an increased PC and PG hydrolytic activity. Another *in vitro* study performed by the same researchers showed that 5 different groups of $sPLA_2$ had the ability to hydrolyze phospholipids and cause surfactant dysfunction.[106] Hydrolysis by group IIA and IID had specificity for PG. Hydrolysis and depletion of PG correlated with surfactant dysfunction. These results in combination with a decreased PG content in asthmatic responders suggested that hydrolysis by $sPLA_2$ and depletion of PG contributes to surfactant dysfunction in asthmatic patients.

Clinical disease

Sputum has been shown to contain surfactant that has a phospholipid composition closely resembling that from BALF.[105] In healthy subjects a close correlation exists between the composition of surfactant isolated from BALF and sputum. Two studies evaluated surfactant in asthmatic patients with naturally occurring clinical disease (showing various degrees of clinical disease) and used sputum analysis to determine surfactant changes.[19,105]

In the first study, sputum was evaluated from patients with acute asthma.[19] During acute asthma, sputum contained increased levels of protein, increased protein/phospholipid ratio, and dysfunctional surfactant. During the recovery phase, protein to phospholipid ratio and surface tension decreased. These findings further suggest that surfactant dysfunction is a feature of the acute asthma attack.

Another study analyzed BALF and sputum from asthmatic patients with clinical disease.[105] In asthmatic patients the proportion of DPPC was decreased in sputum but not in BALF. Similarly, an increased surface tension was only found in sputum but not BALF from asthmatic patients. No significant changes were found in protein content of sputum or BALF of asthmatic patients.

Treatment of asthmatic patients with surfactant

Studies on treatment of asthmatic patients with surfactant are limited and have yielded variable results. In a small randomized controlled trial surfactant versus saline nebulization significantly improved lung function but not blood oxygenation in eleven asthmatic patients.[111] Nebulized surfactant did not improve lung function or bronchial hyperrresponsiveness in mild asthmatic children.[196] Aerosolized surfactant improved lung function in adult patients with stable chronic bronchitis.[197] Another small study indicated that surfactant inhaled as a dry powder decreased the early allergen-induced response in asthma (as evidenced by an improvement in FEV).[112] Natural surfactant administered to asthmatic patients via bronchoscope before allergen challenge (using bronchoscopy) resulted in an increased eosinophilic inflammation following allergen challenge.[198]

Conclusion

In conclusion, surfactant contributes to the normal function of the airways. Animal models of asthma and studies on allergen challenged asthmatics have demonstrated that surfactant dysfunction is present in this disease. However the relationship between surfactant alterations and disease or disease severity remains unclear. In addition, mechanisms leading to surfactant alterations warrant further investigation. As illustrated in this article, surfactant alterations may be caused by a combination of surfactant inhibition, degradation, and impaired

synthesis (see Table I.2.3.). Even though current literature on the efficacy of surfactant treatment in asthmatic patients is limited, some of the preliminary studies have shown encouraging results. Investigation of different surfactant formulations for the treatment of asthma and also in association with currently used asthma medications may yield interesting results in the future.

Table I.2.1.: Cytologic and surfactant changes associated with the guinea-pig model of asthma. The table shows different studies (1-4) with corresponding reference, type of experimental subjects used (Subjects), the type of disease induction (disease), samples collected (BALF or type II alveolar cells), changes in BALF cell count (Cells), protein content, phospholipid content, and method of surface tension assessment (Surface tension).

	Subjects	**Disease**	**Sample**	**Cells**	**Protein**			**Phospholipid**					**Surface tension**	
					Tot	LA	SA	Tot	LA	SA	Ratio	Composition	CapS	PBS
1[13]	Guinea-pig	Acute Challenge	BALF	/	↑	/	/	=	/	/	/	=	/	↑
2[193]	Guinea-pig	Acute Challenge	BALF	↑	=	/	/	↓	/	/	/	/	/	↑
3[14]	Guinea-pig	Chronic Challenge	BALF Type II c	/	↑	/	/	= ↓	/	/	/	/ ↓ PC synthesis	/	↑
4[194]	Guinea-pig	Chronic Challenge	BALF	↑	↑	/	/	/	↑	/	/	↓ PC, ↓ PG, ↑ PI	/	↑

BALF: Bronchoalveolar lavage fluid, tot: total, LA: large aggregates, SA: small aggregates, Ratio: ratio between aggregates, CapS: capillary surfactometer, PBS: pulsating bubble surfactometer, PC: phosphatidylcholine, PG: phosphatidylglycerol, PI: phosphatidylinositol, c: cells, ↑: increase, ↓: decrease, =: unchanged, / not reported or unknown.

Table I.2.2.: Cytologic and surfactant changes associated with human asthma. The table shows different studies (1-6) with corresponding references, type of experimental subjects used (Subjects), the type of disease induction (disease), samples collected (BALF or sputum), changes in BALF cell count (Cells), protein content, phospholipid content, and method of surface tension assessment (Surface tension).

	Subjects	**Disease**	**Sample**	**Cell**	**Protein**			**Phospholipid**					**Surface tension**	
					Tot	LA	SA	Tot	LA	SA	Ratio	Composition	CapS	PBS
1[17]	Asthma	Challenge	BALF	↑	↑	/	/	/				/	↑	/
2[16] **+ 3**[94]	Asthma	Challenge	BALF	↑	↑	/	/	=	/	/	↑ SA/LA	↑ PC/PG ↓ PC (↓ DPPC , ↑ PC w 18:2)	↑	↑
4[18]	Asthma Responders	Challenge	BALF	↑	? =	↑	=	=	=	= (↑)	↓ LA/SA	? = (↑ PC/PG, ↓ PG, ↓ PI)	/	↑
5[19]	Asthma	Acute	Sputum	↑	↑	/	/	**~**	/	/	/	/	/	↑
6[105]	Asthma	Mild-severe	Sputum BALF	= =	= =	/ /	/ /	/ /	/ /	/ /	/ /	↓ DPPC =	?	/

BALF: Bronchoalveolar lavage fluid, tot: total, LA: large aggregates, SA: small aggregates, Ratio: ratio between aggregates, CapS: capillary surfactometer, PBS: pulsating bubble surfactometer, PC: phosphatidylcholine, PG: phosphatidylglycerol, PI: phosphatidylinositol, c: cells, ↑: increase, ↓: decrease, =: unchanged, / not reported or unknown.

Table I.2.3.: Mechanisms of surfactant alterations in asthma or its models.

	Surfactant inhibition	**Surfactant degradation**	**Impaired synthesis**
In vitro	Addition of protein: LOSA	Addition of $sPLA_2$: LOSA Addition of LPL (FA, depletion PL): LOSA	/
Animal model	Ag challenge induces protein infiltration Ag challenge induces LOSA Washing of surfactant restores SA Incubation CLSE with supernatant: LOSA	Repeated Ag challenge: altered PL profile ↓ PC, ↓ PG, ↑ PI	Repeated Ag challenge: ↓ surfactant stores and ↓ synthesis of PC in type II alveolar cells
Human asthmatic	Ag challenge induces protein infiltration Ag challenge induces LOSA Washing of surfactant restores SA Incubation CLSE with supernatant: LOSA	Ag challenge: ↑ levels $sPLA_2$ ↓ PG with dysfunctional surfactant	/

LOSA: loss of surface activity, $sPLA_2$: secretory phospholipase A2, LPL: lysophospholipid, FA: fatty acid, PL: phospholipid, Ag: allergen, SA: surface activity, CLSE: calf lung surfactant extract, PC: phosphatidylcholine, PG: phosphatidylglycerol, PI: phosphatidylinositol, ↑: increase, ↓: decrease, =: unchanged, / not reported or unknown.

Section 3:
Functional Analysis of Lung Surfactant

Numerous methods can be used to evaluate functional properties of lung surfactant.[180] *In vitro* methods allow assessment of surface tension, surfactant film properties, and effect of inhibitors. *In situ* models examine the effect of surfactant depletion and replacement on pressure-volume curves of the lung. The effect of surfactant alterations on pulmonary function, oxygenation status, and surfactant metabolism can be examined *in vivo*, using animal models of disease.

In Vitro methods

This article discusses the most frequently used methods to assess surfactant surface tension. Other *in vitro* methods have been developed for the assessment of surface tension in surfactant films. It is beyond the scope of this chapter to discuss all the methods that are available for surface tension assessment of surfactant. A valuable summary of these methods as well as a more detailed description of the methods explained in this chapter may be found in a recent article by Zuo et al.[1]

Surfactant film analysis

Langmuir-Wilhelmy Balance[1,180]

The Langmuir Wilhelmy Balance (LWB) is widely used to study pulmonary surfactant film behavior.

Surface balances are instruments that measure surface tension-area or surface pressure-area behavior in compressed films (Figure I.3.1.). A Langmuir trough lined with hydrophobic material (e.g. Teflon) contains a liquid subphase. Surfactant film is spread at the air-water interface or forms by adsorption from the subphase. One or more movable barriers allow surfactant film compression or expansion. Changes in surface area are measured and expressed as percentage of initial surface area (without compression) or as an inverse of film concentration in A^{o2}/molecule. A Wilhelmy slide dipped into the liquid surface allows determination of surface pressure by measuring the liquid force exerted on the slide. Surfactant film behavior is

influenced by surfactant concentration and composition, and degree of compression of the film, as well as temperature and humidity.

Advantages

The LWB follows a relatively simple principle. It has the advantage to allow precise determination of surface per molecule because the amount of surfactant spread and available surface area for spreading are controlled. In addition, it allows *in situ* examination of surfactant film structure and other properties by application of molecular biophysical techniques. As described by Y.Y. Zuo: 'Such assemblies allow direct examination of the film molecular structure, orientation, domain formation, topography, electrical surface potential, and localized chemical composition.'[1]

Disadvantages

Large amounts of liquid are necessary to provide a subphase. Compression and expansion rates have to remain slow to avoid surface perturbation and interference with measurement of surface tension. Temperature and humidity influence surfactant film behavior and can be difficult to control. Film leakage or migration is a disadvantage of the LWB. Film migration implies that surfactant at low surface tensions spreads onto surrounding surface and leads to false measurement results. Film migration is avoided by using tightly fitted or Teflon lined instruments. In addition, components of the LWB can be primed with various solutions that help prevent film leakage. In comparison to other *in vitro* models such as the bubble surfactometer, the LWB does not simulate respiration.

Instruments mimicking alveolar conditions

Pulsating Bubble Surfactometer[180,181]

The pulsating bubble surfactometer (PBS) was developed by Enhorning.[199] This instrument allows measurement of surfactant surface tension under conditions that mimic events in the alveoli during breathing (i.e. temperature, humidity, respiratory rate).

A surfactant sample is introduced into a small capillary on a sample chamber (Figure I.3.2.). The sample chamber is placed in the PBS and immersed into a temperature controlled

water bath. A bubble is formed in the sample chamber by pulling air through the capillary. The bubble is drawn out until it is placed at the edge of the capillary at a radius of 0.4 mm. Adsorption is measured for approximately 10 seconds, which is thought to be the time necessary for surfactant phospholipid to reach an equilibrium at the air/liquid interface. The bubble is then pulsated between a minimal radius of 0.4 mm and a maximal radius of 0.55 mm (=50% reduction in bubble surface size). During pulsation, the bubble size can be monitored via a microscope and adjusted to remain within predetermined radii. The pressure gradient across the bubble is measured by use of a pressure transducer and the surface tension is calculated using the Laplace equation $\Delta P = 2\gamma/R$ (P: pressure, γ: surface tension, R: radius). Standard cycling frequency for the PBS is 20 cycles/minute. Cycling rate as well as temperature of the water bath can be adjusted in order to mimic different *in vivo* conditions. For example, cycling rate may be adjusted to represent a higher respiratory rate or temperature may be decreased to mimic effect of cool air on surfactant.

Advantages

The PBS requires very little sample preparation time, analyzes samples within 10 minutes, and sample volume (at a specific concentration) needed is low. Another advantage compared to the LWB is that the PBS simulates conditions in the alveoli (radius, cycling rate, temperature, and humidity). It is possible to analyze surface tension at a higher cycling rate compared to the LWB since the problem of surface perturbation does not occur with the PBS.

Disadvantages

Film leakage or migration (as mentioned with the LWB) may occur along the capillary of the sample chamber and result in falsely elevated surface tension measurement. PBS sample chamber made of Teflon offer a better prevention against film migration compared to the currently available sample chambers made of polyacrylamide. The adsorption time and amount of reduction in bubble surface area are predetermined. Some surfactant samples may need additional time to equilibrate (e.g. in the presence of inhibitors).

At low surface tension the bubble tends to form an ellipse rather than a sphere, therefore, the law of Laplace becomes less accurate for calculation of surface tension. A ‘real-time image

acquisition system' can be added to the PBS and allows analysis of surface tension at any point during pulsation and fitting of the bubble size according to variations in its shape.[200] This system also allows visual detection of film leakage and elimination of data that was acquired during this process.

Captive Bubble Surfactometer[1,180,201]

The captive bubble surfactometer (CBS) was developed by Schurch et al.[201]

An air bubble (1-7 mm diameter) is formed in a surfactant suspension contained in a sample chamber. The air bubble is captured against a hydrophilic roof (i.e. coated with a fixed agar gel support or smooth stainless steel) (Figure I.3.3.). A thin liquid film exists at the air-agarose interface (between bubble and agarose). Bubble volume is controlled by varying the pressure in the sample chamber. A reduction in bubble volume leads to a decrease in bubble surface area, and therefore surface tension. Adsorption can be measured followed by assessment of dynamic surface activity. Surface tension, area, and volume are calculated from bubble height and diameter measured via video image acquisition. Different sizes of sample chambers are available (small and large) that allow assessment of different surfactant concentrations.

Advantages

Film migration does not occur with the CBS since surfactant cannot leak outside the sample chamber. Variations in surfactant concentration within the sample chamber are minimized by subphase stirring. The amount and speed of film compression can be adjusted. Surfactant reservoir behavior, gas transfer properties of surfactant films, and dissolution of anesthetic vapors and gases may be evaluated using the combination of CBS with other techniques.

Disadvantages

It is more difficult to control a specific surface area with the CBS than it is with the LWB, because bubble area depends on its volume and shape. More specifically, bubble compression leads to a decrease in volume and area. This in turn leads to a decrease in surface tension which induces an increase in bubble area. The time for operation, data processing, and

between sample runs is longer with the CBS compared to the PBS. The maximum surfactant concentration that can be used is limited since concentrated surfactant is turbid which limits optical assessment with the CBS.

Methods mimicking airway conditions

Capillary surfactometer

The capillary surfactometer was developed by Liu and Enhorning to study the ability of surfactant to maintain patency of narrow conducting airways.[54]

The capillary surfactometer contains a glass capillary with a small narrowed area (Figure I.3.4.). This section of the capillary has an inner diameter of 0.25 mm which is the approximate width of a human terminal airway.[184] A volume of 0.5 µl of sample fluid is deposited in this section. A constant airflow of 0.3 ml/min is maintained through the capillary. The sample fluid creates a resistance against airflow which results in a progressive increase in pressure. Pressure suddenly drops to zero when the liquid plug is pushed out of the narrow section and air passes freely. If the liquid reaccumulates, the pressure rises again and so on. The capillary surfactometer measures the percentage of time that the capillary remains open to airflow over 2 minutes. If the sample fluid is well-functioning surfactant, the capillary will remain open after the initial pressure rise and drop (100% openness). If the surfactant has poor surface activity, or has a low concentration, or is inhibited by proteins, the capillary will be blocked by fluid repetitively.[54] This frequent closing and reopening of the capillary leads to reduction in the time the capillary is open, which is measured or expressed as the percent openness.

Advantages

The CapS mimics surfactant function in conducting airways and evaluates the ability of surfactant to maintain capillary patency. It requires only a very small sample size (0.5µl) and yields results quickly. The effect of different inhibitors on the ability of surfactant to maintain airway patency can be evaluated.

Disadvantages

The CapS reports the percentage of time that the capillary is maintained rather than a true measurement of surface tension.

Constrained Sessile Drop

The constraint sessile drop (CSD) is a relatively new method that was first developed to determine surface tension and density of polymers.[202] It was later modified for the assessment of pulmonary surfactant.[203]

A drop of surfactant is maintained on a pedestal of stainless steel (Figure I.3.5.).[1] The surfactant drop is in contact with a surfactant reservoir through a hole in the pedestal. The surfactant reservoir is constantly stirred (via a magnetic stir bar). Formation and oscillation of the sessile drop are controlled and humidification of the surrounding air can be measured and adjusted. A gas manifold and chromatograph are in development to allow the possibility to study the action and composition of different gases that may affect surfactant behavior.

Advantages

Film migration (described with LWB and PBS) does not occur with CSD. Compared to the CBS, the CSD allows use of a large range of surfactant concentrations, it is easier to operate, and requires less sample volume. In addition, computed data processing is facilitated by a better contrast between drop and background compared to the contrast between bubble and surrounding surfactant obtained with the CBS. The CSD is able to measure low surface tensions at physiological concentrations.

Disadvantages

Similar to the CBS method, surface tension and surface area are correlated.

***In situ* methods[1,180]**

In situ methods consist in evaluating surface tension within the lung either by analysis of pressure volume curves in excised lungs or using the microdroplet technique.

Excised lung

Mechanical studies of excised lungs provide a reliable model for the study of lung surfactant. Lungs are excised from adult animals, degassed, and reinflated using standardized procedures. The first pressure-volume curve is recorded to define normal lung mechanics. The second curve is recorded following surfactant depletion via lavage, and the third curve is recorded following exogenous surfactant administration. The principle of this method relies on the fact that inflating or maintaining the lung at a fixed volume requires work to overcome tissue and surface tension forces within the lung. In saline filled lungs only the tissue forces are left to overcome. The difference between the P-V isotherms of air and saline filled lungs gives an indication of surface tension forces within the lung.

In vivo **models**[1,180]

In vivo models used for the evaluation of lung surfactant can be subdivided into models of surfactant deficiency used to study the effect of surfactant on NRDS and surfactant dysfunction used to study the role of surfactant in ARDS.

Models of surfactant deficiency

Surfactant deficiency can be studied using premature animals that are 'naturally surfactant deficient', or adult animals that are 'surfactant depleted' via repeated lung lavages.

Premature animals

Small premature animals (e.g. rabbits) are used to study lung compliance during ventilation. In brief, rabbit fetuses are delivered by C-section at 27-28 days of gestation (normal gestational length 31 days). Premature surfactant deficient rabbits are ventilated under controlled conditions. Surfactant is administered via direct endotracheal instillation. The small size of the rabbit allows an easy and even distribution of surfactant within the lung. Measurements can be performed on a large number of animals. However, the small size of premature rabbits does not allow to test different ventilating strategies. Studies of surfactant metabolism are also limited due to a short survival time of these animals.

Large premature animals (e.g. lambs) have a size that is more comparable to human neonates. The premature lamb has been commonly used as a model to evaluate surfactant replacement in NRDS. Lambs at different stages of lung maturation may be used. Lambs delivered by C-section between 120-125 days of gestation (normal gestational length 145-150 days) are profoundly surfactant deficient and die rapidly unless treated with exogenous surfactant and artificial ventilation. Lambs delivered at 132-135 days of gestation can produce enough endogenous surfactant to be maintained for an extended period of time (even without exogenous surfactant administration). The premature lamb model allows thorough assessment of blood oxygenation status and lung function. In addition, different ventilator strategies can be tested and surfactant metabolism may be analyzed. The lamb model is more labor intense and costly compared to the premature rabbit model.

Adult surfactant depleted animals

Surfactant depletion is achieved by repeated lung lavages with saline in animals under mechanical ventilation. Surfactant depletion is confirmed when a drop in blood oxygenation occurs in animals and low surfactant phospholipid content is measured in lavage fluid. The effect of exogenous surfactant on blood oxygenation, lung function, and surfactant metabolism can be studied with this model.

Models of surfactant dysfunction

Acute lung injury (ALI) and surfactant dysfunction can be induced in animal models using different methods: antibody induced, aspiration, bacterial infection/toxin, fatty acid, hyperoxic, in vivo lavage, neurogenic edema and bilateral vagotomy, N-nitroso-N-methylurethane, and viral infection. Assessment of the effects of surfactant therapy and surfactant metabolism is possible with these animal models. It is beyond the scope of this chapter to discuss methods to induce ALI or consequences on the surfactant system in detail. More detailed information may be found in Notter et al.[180]

Conclusion

In conclusion, a number of different methods exist to evaluate lung surfactant function. *In vitro* methods can mimic conditions in the alveoli (PBS) or airways (CapS). Methods such as the

CBS and CSD evaluate surfactant in a bubble or drop, and have the advantage that they are not affected by film leakage. The CSD is a relatively new technique and may allow evaluation of surfactant under a number of different conditions. *In vivo* methods consist of inducing surfactant depletion or injury in animals. Lung function measurements (e.g. lung compliance) give an indication of the effect of surfactant injury and subsequent treatment.

Figure I.3.1.: Schematic representation of a Wilhelmy Surface Balance. A Langmuir trough contains an aqueous subphase with a surfactant film spread at its surface. This surfactant film can be compressed or expanded by action of a movable barrier. Surface tension is measured by forces acting on a Wilhelmy plate in touch with the surface of the surfactant film.
Reprinted from: Biochimica at Biophysica Acta (BBA) – Biomembranes, 1778 (2008), Yi Y. Zuo, Ruud A.W. Veldhuizen, A. Wilhelm Neumann, Nils O. Petersen, Fred Possmayer, Current perspectives in pulmonary surfactant – Inhibition, enhancement and evaluation, Copyright 2008, p 1964, with permission from Elsevier.

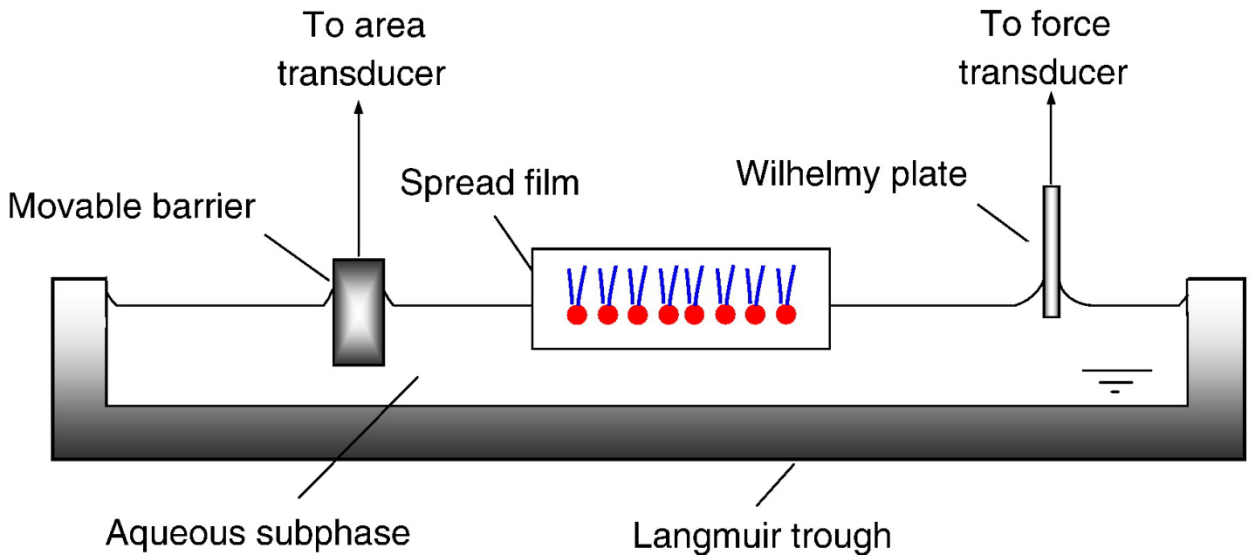


Figure I.3.2.: Schematic representation of a Pulsating Bubble Surfactometer. A sample chamber containing surfactant is placed in the surfactometer and connected to a pulsator. A bubble of 0.4 mm radius is created at the capillary end of the sample chamber by manually drawing in air from the atmosphere. The bubble is pulsated between a minimal radius of 0.4 mm and a maximal radius of 0.55 mm, at 20 cycles per minute, 37ºC. Surface tension is recorded by measuring pressure difference across the bubble surface by the Laplace equation ($\Delta P = 2\gamma/R$). The inset illustrates leakage of surfactant along the capillary portion of the sample chamber.
Reprinted from: Biochimica at Biophysica Acta (BBA) – Biomembranes, 1778 (2008), Yi Y. Zuo, Ruud A.W. Veldhuizen, A. Wilhelm Neumann, Nils O. Petersen, Fred Possmayer, Current perspectives in pulmonary surfactant – Inhibition, enhancement and evaluation, Copyright 2008, p 1965, with permission from Elsevier.

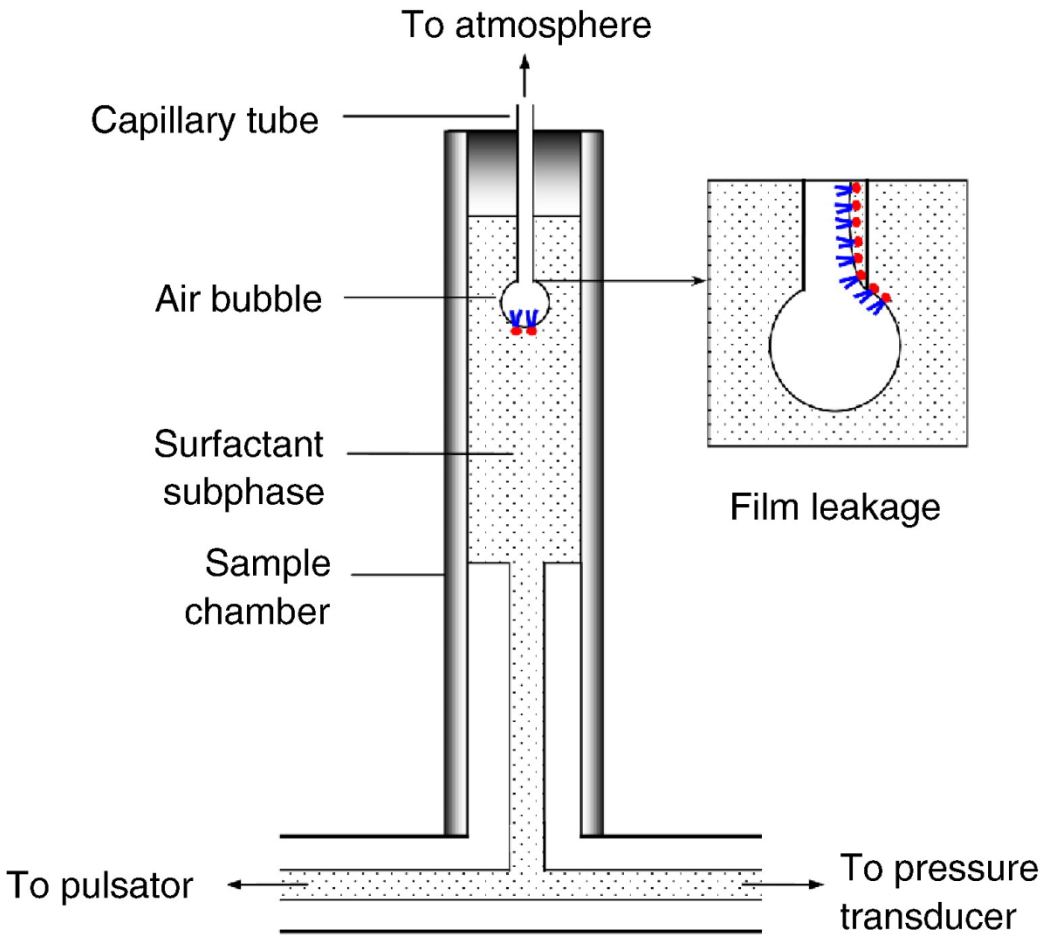


Figure I.3.3.: Schematic representation of a Captive Bubble Surfactometer. A bubble is formed within a surfactant sample. This bubble touches a hydrophobic ceiling. A thin wetting film is maintained between bubble and ceiling. Bubble size is pulsated via a hydraulic pressure control and surface tension is evaluated by bubble shape analysis. Surfactant concentration is maintained constant in the sample chamber by action of a stir bar. The inset shows possible multilayer structures of surfactant at the air-liquid interface.

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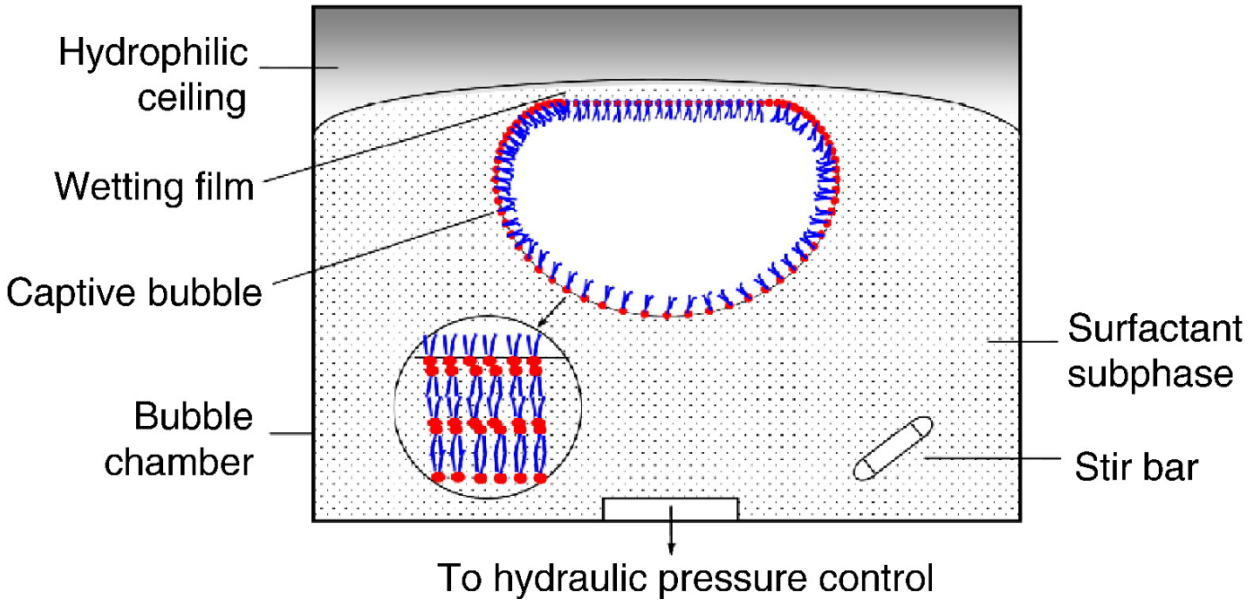


Figure I.3.4.: Schematic representation of the setup of a Capillary Surfactometer. Surfactant to be examined is placed in a small narrowed section of a glass capillary. Pressure at one end of the capillary is raised, measured (with a pressure transducer), and recorded. The liquid column is pushed out of the narrowed section. Depending on the nature of the liquid, it may reaccumulate or leave the capillary open. A camera allows to assess pictures of fluid column behavior in the capillary.

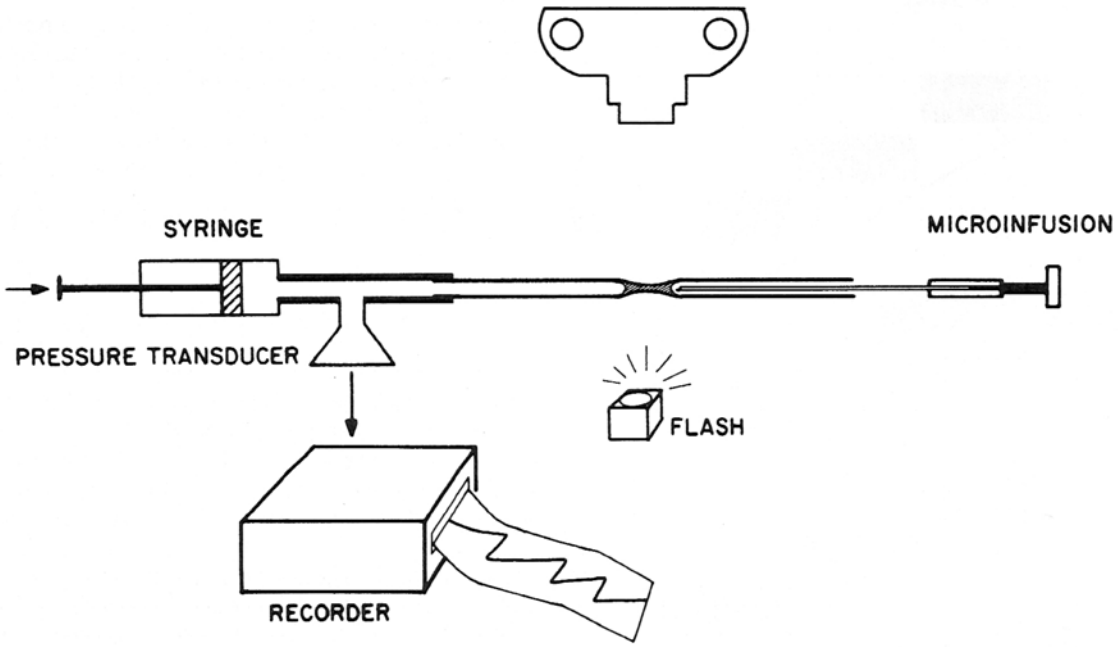
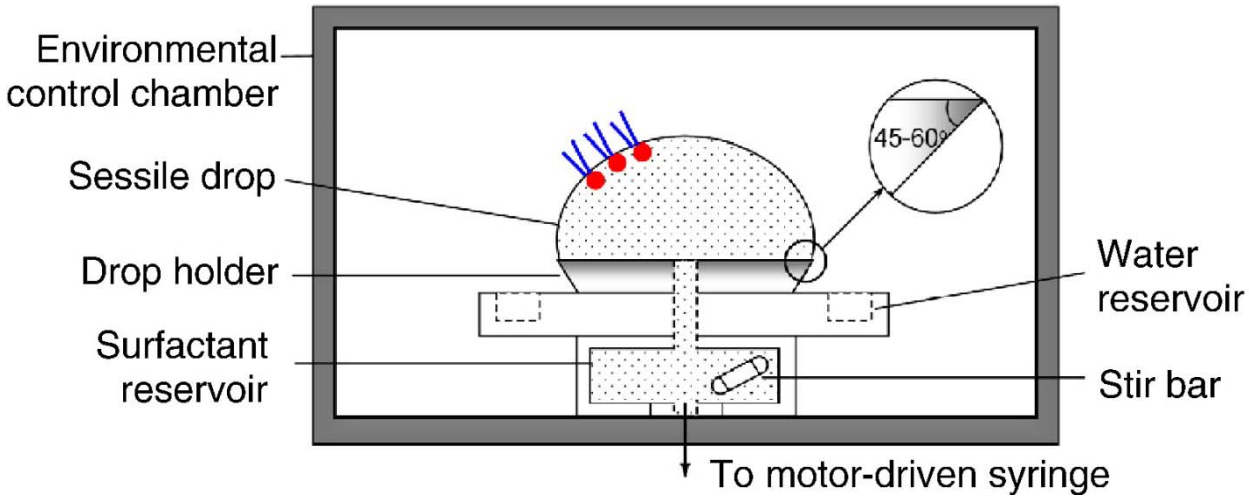


Figure I.3.5.: Schematic representation of a Constraint Sessile Drop. A drop of surfactant (sessile drop) is constrained on top of a pedestal (drop holder) with sharp edges. The drop of surfactant communicates with a surfactant reservoir that is constantly stirred. A motor driven syringe allows adjustment of drop size on top of the pedestal and pulsation. Surface tension is evaluated by drop shape analysis. The drop is contained within an environmental chamber where temperature, humidity, and gas composition can be adjusted.

Reprinted from: Biochimica at Biophysica Acta (BBA) – Biomembranes, 1778 (2008), Yi Y. Zuo, Ruud A.W. Veldhuizen, A. Wilhelm Neumann, Nils O. Petersen, Fred Possmayer, Current perspectives in pulmonary surfactant – Inhibition, enhancement and evaluation, Copyright 2008, p 1969, with permission from Elsevier.



Section 4:
Review of Equine Recurrent Airway Obstruction

Recurrent airway obstruction (RAO) is an important cause of chronic pulmonary disease in mature horses.[204] RAO has been described by a number of different names including chronic obstructive pulmonary disease (COPD) and heaves.[205] As a result of the 'International Workshop on Equine Chronic Airway Disease' in 2000, the name 'Recurrent Airway Obstruction' was recommended to describe this disease and to emphasize its distinction from human COPD.[9]

RAO develops following exposure of susceptible horses to barn environment and hay feeding.[205-208] Affected horses typically develop coughing and various degrees of airway obstruction (as indicated by nostril flaring and increased expiratory effort). RAO is characterized by chronic neutrophilic airway inflammation with mucus accumulation and bronchial hyperresposiveness.[208,209] Remission from clinical disease may be achieved for some horses through modification of their environment by minimizing exposure to respirable dust.[205-208]Airway obstruction in RAO is responsive to administration of bronchodilators and corticosteroids.[205-208]

Classical RAO is a disease of stabled horses exposed to barn environment and more specifically hay.[205-208] Affected horses typically enter remission upon turn out to pasture. Depending on the housing conditions the disease may appear seasonal for horses stabled during the colder months of the year. Summer-pasture-associated obstructive pulmonary disease (SPAOPD) appears to be the 'pasture equivalent' of RAO.[210,211] Clinical signs develop preferentially during the summer time and more often in warm and humid climates.[210,211] Mixed forms of RAO and SPAOPD have also been described.[205] Clearly, SPAODP and mixed disease are more challenging to treat since elimination of the 'disease causing' environment typically requires horses to be moved to another geographic region.[206]

Etiology

Both RAO and SPAOPD occur upon exposure to environmental factors that induce clinical disease.[205] A number of factors have been implicated in the etiology of RAO, most of which may be contained in organic dusts arising from hay and bedding. Molds (*Faenia*

rectivirgula, *Aspergillus fumigatus*, *Thermoactinomyces vulgaris*), endotoxin, forage mites, plant debris, inorganic dust components, and noxious gases all potentially contribute to the development of airway inflammation in RAO horses.[205,212-215] Molds and endotoxins have both a strong potential to cause airway disease but challenge with either of these factors used separately did not induce an airway inflammation and obstruction that was as pronounced as that by natural hay challenge.[213-216] It is likely that the above mentioned factors act in concert to induce airway disease.[207]

Risk factors and genetic predisposition

A recent study analyzed risk factors for RAO from 1444 cases evaluated over a period of 9 years.[217] This study showed that increasing age was associated with an increased risk to develop the disease. Horses over 7 years of age, also historically considered to be more frequently affected, were 6-7 times more likely to develop RAO. In addition, thoroughbreds, ponies, and mares were more likely to develop the disease, findings that have not been demonstrated in previous reports.[218] This report also indicated that horses were more likely to receive veterinary care during winter and spring.[217]

A genetic predisposition for RAO has long been suspected. At least 2 studies have shown that offsprings resulting from mating between horses that had been diagnosed with RAO were more likely to be diagnosed with RAO than those where only one or none of the parents had the disease.[219,220] Recently, a region on chromosome 13 coding for IL-4 receptor A chain was linked to RAO in one family of warmblood horses derived from the same stallion.[221]

Clinical signs and examination

Clinical signs of RAO vary from very mild signs (occasional coughing and exercise intolerance) to severe respiratory distress.[205-208] Chronic coughing, with or without nasal discharge, and increased expiratory effort are typical clinical signs.[205-208] Coughing occurs upon exposure to dust, at the beginning of exercise, at rest, and can be easily induced by palpating the trachea of affected horses. Nasal discharge (serous to mucopurulent) may be intermittent and sometimes more noticeable after exercise. Horses with RAO are less exercise tolerant and need a longer time to recover from exercise.[222] Nostril flaring and abdominal push are both indicators of

airway obstruction and have been used in scoring the degree of respiratory effort (see table I.4.1.).[223] Prolonged, untreated disease leads to hypertrophy of the external abdominal oblique muscle (heave line).[205-208] Tachypnea, and tachycardia develop with hypoxemia. Severe respiratory distress may result in weight loss and inability to perform even minor exercise.[224] Pulmonary auscultation reveals increased bronchovesicular sounds, wheezes, and crackles all of which are exacerbated with the use of a rebreathing bag.[9,206]

Table I.4.1.: Clinical scores used for evaluation of RAO horses.

Individual scores	**Attribution criteria**
Abdominal expiratory effort	
1	*No abdominal component to breathing*
2	*Slight abdominal component*
3	*Moderate abdominal component*
4	*Severe, marked abdominal component*
Nostril flaring	
1	*No flaring*
2	*Slight, occasional flaring of nostrils*
3	*Moderate nostril flaring*
4	*Severe continuous flaring during each respiration*
Total scores	***Clinical signs of RAO***
2	*No signs*
3-4	*Mild signs*
5-6	*Moderate signs*
7-8	*Severe signs*

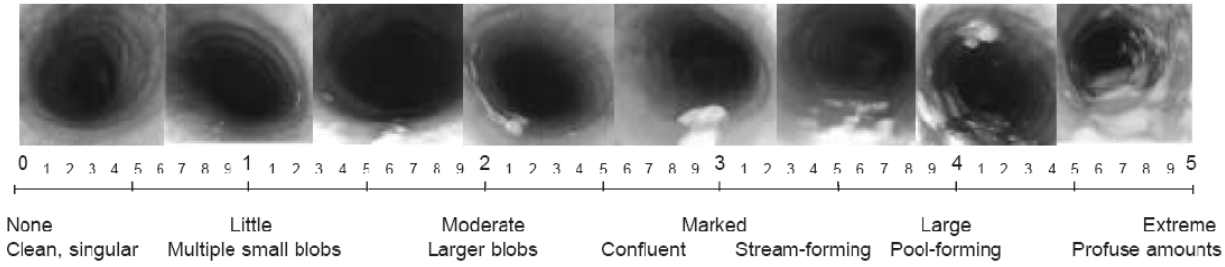
Diagnosis

Diagnosis of RAO is, in part, based on history of clinical signs (see above) that develop with either exposure to barn environment (RAO) or exacerbation during the summer months (SPAOPD).[205-208]

Typically, complete blood cell counts (CBC) and blood chemistry analyses are within normal limits in RAO horses. Arterial blood gas analyses reveal hypoxemia that is related to disease severity.[205-208] Hypercapnia is infrequent and normo- or hypocapnia are more often present caused by hyperventilation.[205-208]

RAO is characterized by an excessive mucus accumulation evident during endoscopic examination of the trachea. Gerber et al. developed a scoring system (scores 0-5) to quantify the amount of tracheal mucus (see Figure I.4.1.).[225]

Figure I.4.1.: mucous scores (0-5) used to describe amount of mucus present in tracheal endoscopic examination. Reprinted from: Equine Veterinary Journal (EVJ), 36 (7), page 577. 1778, 2004. Gerber V, Straub R, Marti E, Hauptmann J, Herholz c, King M, Imhof A, Tahon L, Robinson NE. Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. Copyright 2008, with permission from EVJ.



During endoscopic examination, RAO affected horses tend to cough more easily than healthy horses and examination of the bronchial bifurcation often reveals mucosal thickening in RAO horses. However, scoring tracheal septum thickness scores do not correlate with clinical severity, endoscopic or cytological findings in RAO horses.[226]

Initial confirmation of RAO may include evaluation of airway fluid obtained by tracheal aspiration (TA). Sterile technique is used to acquire this fluid, permitting cytological examination and culture of aspirated material to distinguish between septic and non-septic airway inflammation. TA from RAO horses are reported to contain minimal bacteria associated with airway neutrophilia.

Bronchoalveolar lavage fluid (BALF) collection is another diagnostic procedure used to evaluate horses with suspected RAO. Examination of BALF cytology may also be used to monitor response to treatment in horses where the diagnosis of RAO has already been established. Neutrophilia on BALF cytology is a characteristic feature of clinical RAO.[9,205-208] Neutrophils are present in only low numbers (i.e. < 5%) in airways of healthy animals.[227] RAO horses in clinical remission can have a low or slightly elevated neutrophil percentage.[228]

Exposure to moldy hay leads to a rapid increase in BALF neutrophil percentages in RAO horses.[229] BALF neutrophil percentages return back to remission values if RAO horses exposed to short-term challenge environment return to a dust reduced environment.[230] Prolonged exposure to barn environment may have a prolonged effect on BALF neutrophil percentage.[227] BALF neutrophilia also occurs in some 'healthy' horses without signs of airway disease when they are exposed to barn environment.[231,232] As compared to samples from RAO horses, BALF neutrophilia is usually moderate in horses that do not have RAO.[231]

Conventional lung function testing (LFT) allows, amongst other parameters, measurement of maximal change in pleural pressure during tidal breathing(maxΔPpl), pulmonary resistance and dynamic lung compliance.[233] LFT is often applied in research settings for an objective evaluation of the degree of airway obstruction and adds to the ability to detect airway obstruction that would not be diagnosed by simply evaluating clinical scores.[223] Typical changes of LFT in RAO horses include increased maxΔPpl, increased pulmonary resistance, and decreased dynamic lung compliance.

Several other techniques have been developed to assess lung function in RAO horses. These include: forced oscillatory mechanics, flowmetric plethysmographic method, forced expiratory maneuvers, and bronchoprovocation tests.[233] Some of these techniques (e.g. flowmetrics) are less invasive, making them more applicable for use in field conditions. The forced expiration technique (developed as a modification of forced expiratory volume used in human medicine) is capable of detecting mild changes in pulmonary function but requires sophisticated equipment (including a powerful vacuum pump that enables an induced 'forceful expiration' in a heavily sedated horse) only available in a few equine referral hospitals.[234] Bronchoprovocation tests, using histamine or methacholine, are used in horses without visible airway obstruction to assess potential airway hyperreactivity.[233]

Pathophysiology of RAO

RAO develops following exposure of susceptible horses to organic dust and is characterized by airway inflammation, mucous accumulation, and bronchoconstriction.

Inflammation

Exposure to organic dust leads to a rapid increase in BALF neutrophil percentages in RAO horses.[229] The inflammatory reaction in RAO depends on the action and interaction of a number of cells including neutrophils and lymphocytes but also other cells found in the lung such as epithelial cells, mast cells, macrophages, and their mediators.

Neurophilic airway inflammation in RAO horses may be a non specific inflammatory response of the lung in response to inhalation of a variety of agents.[227] Mechanisms of neutrophil recruitment and trafficking through the lung depend on a large number of adhesion molecules (i.e. ICAM-1), chemoattractants (i.e. macrophage inflammatory protein MIP, IL-1β, TNFα, IL-17), and cells (i.e. endothelial cells, macrophages).[227,235-239] Interleukin 8 (IL-8) is a neutrophil chemoattractant produced by a number of pulmonary cells including neutrophils. IL-8 levels are increased in BALF and its expression is increased in bronchial and BALF cells from RAO horses.[205,235,237,238,240-242] Transciption factor nuclear factor κB (NF- κB), an important regulator of inflammatory genes, is highly active in equine bronchial and BALF cells during RAO crisis.[243-245] In RAO horses NF- κB is composed of p65/p65 homodimers rather than of p50/p65 heterodimers found in healthy horses.[243,245] These p65/p65 homodimers induce IL-8 chemoattractant and lead to neutrophilic airway inflammation whereas p50/p65 heterodimers are known to induce eosinophil chemoattractant. Neutrophils are normally cleared from the airways via apoptosis and phagocytosis by macrophages.[230] One study provided evidence for delayed neutrophil apoptosis as a contributing factor for airway neutrophilia.[246]

Lymphocyte T helper cells (Th1 and Th2) are modulators of the immune response. Classically Th1 cells secrete IL-2, lymphotoxin, and IFNγ that induce natural killer lymphocyte activated killer cells, effector cytotoxic T lymphocytes, activate macrophages, and prime neutrophils.[247] Th2 cells secrete IL-4 and IL-5 and lead to B cell differentiation to plasma cells, activation of mast cells, and eosinophil recruitment/activation.[247] It has been suggested that pulmonary immune response, including that of the horse, is not necessarily strictly polarized towards a Th1 versus Th2 type response.[248] In RAO horses a cytokine profile typical for Th2 cells and thus allergic disease has been described.[11,249] Increased expression of IL-4 and IL-5 mRNA and decreased expression of IFNγ, (evidenced by *in situ* hybridization) develops rapidly

in RAO horses exposed to organic dust, persists with continued exposure, and wanes with remission.[11,249] However, increased expression of both IL-4 and IFNγ has also been described in RAO horses.[238,240] Yet others have found no alteration in lymphocyte cytokine expression characteristic of either a Th1 or Th2 response.[250]

An imbalance in the oxidant/antioxidant status is implicated in the pathophysiology of pulmonary inflammation, including RAO. The lung is continuously exposed to oxidants (or reactive oxygen species, ROS) from inhaled air (i.e. ozone, ultra-fine particles, endotoxin) but also to endogenous oxidants such as those liberated by phagocytes (i.e.myeloperoxidase by neutrophils and nitric oxide by macrophages). In RAO horses during crisis, an increase in the reduced form of glutathione (GSH) and the ratio between GSH and the sum of oxidized and reduced glutathione (GRR) indicates an increase of antioxidants in the face of oxidative stress.[251] Levels of 8-isoprostane (a lipid peroxidation byproduct) were increased in BALF from RAO horses in crisis.[252] Myeloperoxidase (a potent oxidant) levels were higher in BALF from RAO horses in crisis and remission compared to controls.[253] Even though several of the previously mentioned components correlated with measures of lung function in RAO horses, very little is known on their exact effect within the lung.[254]

Mucus accumulation

Mucus accumulation is a non-specific sign for airway inflammation. According to Rogers et al. '…mucus secretion appears to be such a fundamental homeostatic process, virtually all regulatory and inflammatory mediators and interventions that have been investigated increase secretion acutely.'[255] Excessive mucus accumulation may be due to either decreased muco-ciliary clearance or increased production/secretion of mucus.[256]

Histopathological evaluation of respiratory epithelium in the RAO-affected horse showed a decrease in the number of ciliated epithelial cells, although *in vivo* effects of these findings are unknown.[257] Muco-ciliary clearance is largely affected by mucus viscoelasticity (term used to describe liquid- and solid-like properties of mucus) but also by mucus hydration and surface properties (also described as adhesivity and wettability) .[258] Increased mucus viscoelasticity was found in RAO-affected horses 24 hours following exposure to challenge environment.[259,260] Increased mucus viscoelasticity in RAO may be due to accumulation of inflammatory products

and debris,[261,262] or elevated levels of specific mucins (α 1,2 fucose or N-acetyl glusoamine) demonstrated in RAO horses.[263] Adhesivity or wettability of mucus is believed to be influenced by its phospholipid content derived from surfactant.[258,264] Exogenous surfactant therapy has been shown to improve muco-ciliary clearance in several animal species, including the horse.[56,258,264]

Several studies have provided evidence for increased mucus production and secretion in RAO horses. RAO-affected horses have an increased number of goblet cells associated with increased storage of mucosubstances.[257,265] Mucous cells from RAO-affected horses that are recovering from acute disease express Bcl2, an apoptosis inhibitor.[266] It is unknown whether Bcl2 prolongs bronchial mucous cell life in RAO horses. Overexpression of the equine soluble secreted glycoprotein (eCLCA1) in RAO-affected horses was explained by goblet cell hyperplasia and metaplasia rather than transcriptional upregulation.[267] When compared to normal horses, a higher mucin gene expression (MUC 5AC) was reported in RAO horses and was proposed as a cause for mucous overproduction.[268] Recent evidence suggests that neither up-regulation of the inflammatory cytokines (IL-13, and IFN-γ) nor epithelial genes (eCLCA1, Bcl2, MUC5AC, EGFR) is associated with increased mucus accumulation in chronic RAO.[269]

Airway hyperresponsiveness and bronchoconstriction

Smooth muscle contraction in the airways depends on a balance between factors that favor bronchoconstriction versus those that promote bronchodilation. Postganglionic parasympathetic nerve fibers liberate acetylcholine (ACh) which binds to M3 receptors on smooth muscle, causing release of intracellular calcium, and contraction.[270] This pathway is enforced by binding of ACh to M2 receptors, inhibition of cyclic adenosinemonophosphate (cAMP), which also supports muscle contraction. Conversely, binding of epinephrine (released by the adrenal gland) to β2 adrenoreceptors increases cAMP production and induces bronchodilation. Release of NO from parasympathetic nerve fibers increases production of cyclic guanosinemonophosphate (cGMP) and muscle relaxation. The non-adrenergic-non-cholinergic nervous system (iNANC –inhibitory and eNANC-excitatory) also supplies innervations to the airways. The iNANC (responsible for bronchorelaxation by liberation of NO) is dysfunctional in RAO horses.[271,272]

A series of inflammatory mediators liberated in association with RAO promote bronchoconstriction.[273-275] Bronchoconstriction in RAO horses is easily induced in response to different inhaled or locally released stimuli.[206] This hyperresposniveness is the strongest during crisis but is also easily induced while horses are in remission. Causes of airway hyperresponsiveness include inflammation,[273-275] smooth muscle hypertrophy,[206,276] and dysfunctional iNANC system (see mechanisms mentioned above).[271,272]

Pathology

Macroscopic changes in the lung and respiratory tract include mucus accumulation and mild pulmonary hyperinflation.[206] At the microscopic level, pathological changes are found in conducting airways, terminal airways and alveoli of RAO horses.[257,277] In conducting airways both ciliated and goblet cells show histological alterations.[257] Ciliated cells are replaced by undifferentiated cells or show loss or malformation of cilia. Goblet cell metaplasia develops with increased accumulation of mucosubstance.[257,265] Peribronchial inflammatory cell infiltration consists mostly of mononuclear cells.[257] Dilated intercellular clefts and accumulation of mast cells is also present.

A number of focal changes are found in the terminal airways of RAO horses. Exudate and mucus accumulate in the bronchioli and sometimes extend to the alveoli.[277] Clara cells decrease in numbers, undergo structural changes (degeneration and lack of differentiation), and are progressively replaced by goblet cells (the latter are usually not found at this level in healthy, younger horses). Inflammatory cells accumulate in the peribronchiolar region and in severe cases fibrosing peribronchiolits develops. Submucosal fibrosis often develops and smooth muscle hyperplasia may be seen.

Focal changes in the alveolar region include: alveolar hyperinflation and fibrosis. Early alveolar fibrotic changes are found in a majority of RAO horses and often in association with bronchiolar lesions.[277] Type I alveolar cells may be increasingly replaced by type II alveolar cell in fibrotic alveoli. Alveolar type II cells may undergo degeneration and necrosis. Ultrastructurally these cells may show ‘crater-like secretory orifices’ on their surface whereas others are characterized by an increased number and size of lamellar bodies.[277] In addition, peribronchial cysts may occur filled with lamellated contents believed to be surfactant. Both size

and number of these cysts may be associated with disease severity and degree of peribronchiolar changes.

Conclusion

RAO is a chronic pulmonary disease in mature horses that shares certain similarities with human asthma.[209] Disease development occurs upon exposure to potential allergens and clinical signs include coughing, reversible airway obstruction, and wheezing. Pathophysiology is characterized by inflammation, mucus accumulation, and bronchial hyperresponsiveness. Clinical signs respond to eviction of potential allergens and treatment with bronchodilators and corticosteroids. A key difference between the RAO and asthma includes, neutrophilic versus eosinophilic airway inflammation. However, a Th2 type cytokine profile has been described in RAO horses and airway neutrophilia occurs in many patients with severe asthma.[11,278]

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CHAPTER II:

ANALYSIS OF LUNG SURFACTANT (PHOSPHOLIPID) COMPOSITION AND FUNCTION

This chapter gives an overview of the methodology used for surfactant analysis. Chapters III, IV, and V contain a summarized description of all the methods used for each project. More detailed information about methods can also be found in the appendix of this thesis.

Bronchoalveolar lavage fluid (BALF) collection

Horses were restrained in stalls and provided sedation by intravenous administration of butorphanol (Fort Dodge, Fort Dodge, IA) (0.01 mg/kg) and detomidine (Pfizer Exton, PA) (0.01 mg/kg). After applying a nose twitch a bronchoalveolar lavage (BAL) tube (Bivona, Gary, IN) was passed through the nasal passages and into the trachea. Local anesthetic (0.4% lidocaine) was administered when horses started coughing. The BAL tube was advanced until wedged and the cuff was inflated with 6 ml of air. Three aliquots of 100 ml prewarmed sterile saline solution were infused and reaspirated. The aspirated fluid was mixed and pooled in a sterile specimen cup maintained on ice. Recovered fluid volume and quality (i.e. amount of foam, presence of mucus) were recorded. Visible strands of mucus, present in some of the samples, were aspirated by hand with a pipette. BALF was not filtered through gauze since the latter may reduce the amount of surfactant recovered. BALF samples were processed within 30 minutes after collection in order to minimize sample degradation.

BALF was centrifuged at 400 x g, 4ºC, for 10 minutes to pellet cellular components. Cell-free supernatant was aspirated with caution, separated into aliquots, and stored in 15ml cryovials. Cell-free BALF was stored at -80ºC until extraction and analysis could be performed. Samples were frozen because it would have required a minimum of 4 hours to isolate surfactant according to the methods that we used (see below). Since we sampled between 4-8 horses per day, it would not have been possible to process all samples without additional personnel. Freezing of samples before surfactant isolation and analysis has been described by other groups.[1,2]

Surfactant isolation

For surfactant isolation, cell-free BALF supernatant was centrifuged at 40,000 x g for 1 hour, which allowed to separate it into crude surfactant pellets (CSP) and supernatant (Supe). The supernatant (Supe) was the non-pelleted fraction of cell-free BALF following ultracentrifugation. The remaining CSP was washed two times with 100 ml of normal saline (pH 7.4) and was resuspended in a known volume of normal saline.

In the literature, surfactant recovered from BALF is described as having 2 different fractions: large aggregates (LA) and small aggregates (SA).[2,3] These 2 fractions are the result of *in vitro* separation of BALF into a pelleted and non-pelleted fraction following centrifugation. It is believed that in the normal lung, alveolar surfactant consists primarily of dense, LA forms that are enriched in surfactant proteins and tubular myelin. Large aggregates (LA) are surface active, spreading rapidly to reduce surface tension at the air-liquid interface. In contrast, SA are relatively depleted of surfactant proteins and do not reduce surface tension actively at the air-liquid interface. SA phospholipids are thought to represent surfactant forms destined for catabolism or recycling. The ratio of large to small aggregate forms (LA/SA) is critical to maintaining surfactant function in health and disease.

Methods used in different studies to process samples and isolate surfactant have varied widely (for a review of different methodologies see article by Griese et al.[2]). For example, ultracentrifugation of cell-free BALF has been done with and without prior storage of samples at temperatures between -20°C to -80°C. Ultracentrifugation has been performed at different speeds and for a variable length of time. Finally, ultracentrifugation was accomplished either simply using cell-free BALF or by using a density gradient. Because of this variety in methods used for surfactant isolation or separation it is important to report methods used. Comparison of results between studies must be made bearing in mind differences in methodology. While some authors accept the use of the terminology LA and SA across studies,[2] other have pointed out that it may be more appropriate to use the terms CSP and Supe in cases where cell-free BALF samples were frozen prior to ultracentrifugation.[1] The latter is the terminology that we adopted throughout this thesis.

Sample extraction and quantitation of total phosphorus

Sample extraction: Bligh and Dyer Method

The two-step Bligh and Dyer method was used for lipid extraction from our BALF samples. It represents the most frequent method used for lipid extraction of liquid samples.[4,5] All samples were run in triplicates and results were accepted when the coefficient of variation was less than five percent.

Extraction was initiated by combining an aqueous sample (e.g. 5 µl of CSP and 495 µl saline or 1000 µl of Supe) with a chloroform/methanol mixture (v/v 1:2.5:1.25). This mixture was vortexed thoroughly. After the addition of more chloroform and water, the sample was centrifuged to separate lower (organic) and upper (aqueous) phases in the test tube. The organic phase contained the lipid fraction, and was collected using a Pasteur pipette. The aqueous phase was washed with an additional volume of chloroform and centrifuged, and the lower phase was collected again. The two organic phases were combined, and dried under a continuous stream of nitrogen gas.

Quantitation of total phosphorus

Standards of KH_2PO_4 were prepared in triplicates (0.025mM to 5mM). Standards and samples were digested with perchloric acid at 170-190ºC for 25 minutes. Ammonium molybdate was added to samples/standards, and reacted with phosphate compounds in the sample/standard to form phosphomolybdic acid which was reduced to molybdenum by addition of ascorbic acid. Tubes were incubated in a 50ºC water bath for 15 minutes. Determination of phosphorus content was completed by using a spectrophotometric method and a standard curve.

Phospholipid compositional analysis

The methods chosen for phospholipid compositional analysis depend on the type of information needed. Generally speaking thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) allow separation of the main phospholipid classes. Mass spectrometry (MS) can be used to identify different molecular species within a phospholipid class. We choose to use HPLC separation of phospholipid classes since this method was readily

available and was previously described for analysis of lung surfactant phospholipid composition.[6,7] In addition, compared to MS, it was cheaper to use, required less technical expertise, and required standardization for the major phospholipid classes only. MS requires extensive standardization for each phospholipid molecular species.

Methodology

Samples (CSP) were extracted according to methods described above. After extraction, the organic phase was dried under N_2, resuspended in chloroform, and analyzed for phospholipid content.[8] Phospholipid composition of CSP was determined by separation of individual phospholipids with high performance liquid chromatography (HPLC) using a Kromasil silica column (Drachrom; Greensboro, NC) and a dual solvent system gradient from 100% Mobile Phase A ($CHCl_3$:MeOH:NH_4OH; 80:19.5:0.5) ramped to a maximum of 25% Mobile Phase B (MeOH:H_2O:NH_4OH; 80:19.5:0.5).[7] Quantitation of individual phospholipid peaks from the HPLC eluent was performed with an evaporative light scatter detector (SEDERE; Alfortville, France). Retention times and binomial response characteristics of individual phospholipids and lysophospholipids were defined by use of commercially available standards (Avanti Polar Lipids, Alabaster, AL). This sensitive method measured individual phospholipids in the range of 0.1 to 4.0 nmoles and required a minimum surfactant sample size of 12 nmoles. To calculate absolute phospholipid concentrations, a fixed concentration of phosphatidylbutanol (PB) was added to a known concentration of sample after lipid extraction in order to serve as an internal standard.

The amount of the following major phospholipid classes was determined: Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Sphingomyelin (SPH).

Surface activity evaluation with the pulsating bubble surfactometer (PBS)

A pulsating bubble surfactometer (PBS) (General Transco; Largo, FL) was used to measure surface tension lowering activity of CSP.[9] Surfactant from the CSP fraction (40 μl) was used at a final concentration of 0.5 mg phospholipid/ml in buffered saline containing 1.5 mM $CaCl_2$, and was analyzed at 20 cycles/min for 10 min at 37°C in the surfactometer. Results were

reported as γ_{min}, which represented as the minimum surface tension achieved over the 10 minute period.

The PBS was developed by Enhorning[9] and is used extensively to assess the surface tension lowering ability of lung surfactant. The PBS allows assessment of surface tension at physiologically relevant cycling rates (20 cycles/min), area change (50%), temperature (37°C), and humidity (>95%). In the PBS, a small air bubble is formed in an aqueous dispersion of surfactant held in a plastic chamber. The bubble which communicates with ambient air is then oscillated at a known rate between minimum and maximum radii of 0.4 and 0.55, respectively by means of a precision pulsator that moves liquid in and out of the sample chamber. The bubble is monitored through a microscope to ensure that minimum and maximum sizes remain constant throughout an experiment. Pressure in the liquid during pulsation is measured by a transducer and surface tension (γ) is calculated according to the Laplace equation for a sphere:

$$\Delta P = \frac{2\gamma}{R}$$

ΔP: pressure difference γ: surface tension

R: bubble radius

We choose to use the PBS for evaluation of surfactant function. Even though the PBS mimics conditions in the alveoli, rather than airways, it is generally considered reliable to distinguish functional from dysfunctional samples.

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CHAPTER III:

SURFACTANT IN HEALTHY HORSES: EFFECTS OF AGE AND BRONCHOALVEOLAR LAVAGE FLUID CHARACTERISTICS

ABSTRACT

Background: Surfactant alterations are described in horses following exercise, anesthesia, prolonged transport, in horses with recurrent airway obstruction, and in neonatal foals. The effect of horse age or bronchoalveolar lavage fluid (BALF) sample characteristics on surfactant is unknown.

Objectives: To evaluate surfactant phospholipid composition and function in healthy horses, and to investigate the influence of age and BALF sample characteristics on surfactant.

Animals: Seventeen healthy horses 6 to 25 years of age maintained on pasture year round.

Methods: BALF was collected under sedation using standard procedures and was assessed for recovery volume, nucleated cell count, and cytology. Cell-free BALF was separated into crude surfactant pellet (CSP) and surfactant supernatant (Supe) via ultracentrifugation. Phospholipid and protein content were determined from both of these fractions. CSP phospholipid composition was analyzed using HPLC with an evaporative light scatter detector. Surface tension of CSP was evaluated with a pulsating bubble surfactometer. Correlation analysis was used to evaluate associations between age, BALF sample characteristics and surfactant variables.

Results: Increasing age was associated with decreased phospholipid content in CSP ($r = -0.6338$, $p = 0.0112$) but not Supe ($r = -0.3706$, $p = 0.1738$). Age did not affect protein content of CSP ($r = -0.4928$, $p = 0.0620$) or Supe ($r = -0.1146$, $p = 0.6843$), or surfactant phospholipid composition or function. Age-related surfactant changes were unaffected by BALF recovery percentage, nucleated cell count, and cytological profile.

Conclusions and Clinical Relevance: Older horses have decreased surfactant phospholipid content which might be due to age related pulmonary changes. Surfactant composition is unaffected by BALF sample characteristics at a BALF recovery percentage of at least 50%.

This article (Chapter III) is planned to be submitted in a modified form to the Journal of Veterinary Internal Medicine (JVIM).

Introduction

Pulmonary surfactant lowers surface tension (biophysical function) at the level of the alveoli and airways and modulates pulmonary immune responses (immunological function).[1-3] A large portion of surfactant (>80%) is composed of phospholipids which are largely responsible for its biophysical function. Surfactant contains approximately 12% of the following surfactant proteins: SP-A, SP-B, SP-C, and SP-D. SP-A and SP-D are part of the innate immune defenses of the lung,[4] whereas SP-B and SP-C contribute to biophysical surfactant function.[5] Surfactant also contains a small percentage of neutral lipids (i.e. cholesterol, triglycerides), the role of which is not fully characterized. Surfactant is synthesized, stored, and excreted by type II alveolar cells.[6] The majority of surfactant undergoes extensive recycling; a small part of surfactant is moved towards the airways, and another fraction is degraded via alveolar macrophages.[7,8]

Surfactant alterations play a role in alveolar and airway diseases. In the alveoli, low surfactant surface tension is essential to prevent alveolar collapse.[1,3] Surfactant deficiency present in premature animals with immature lungs leads to neonatal respiratory distress syndrome.[9] Surfactant dysfunction, evident as high surface tension, contributes to the pathophysiology of acute respiratory distress syndrome.[10,11] In the airways, surfactant helps maintain patency of small airways,[12] improves muco-ciliary clearance,[13] and decreases bronchoconstriction in response to inhaled allergens.[14,15] Alterations in surfactant composition and function have been described in a variety of airway diseases.[1,16] In human medicine surfactant alterations also occur in patients with allergic asthma,[15,17,18] cystic fibrosis,[19] chronic bronchitis,[20] and viral respiratory diseases.[1,3] Surfactant alterations have been reported in horses following transport,[21,22] exercise,[23] general anesthesia,[24] as well as in horses with recurrent airway obstruction,[25,26] and neonatal foals.[27]

Surfactant changes associated with pulmonary maturation are well documented in neonates from a number of animal species,[28-30] including the horse.[27] However, only a few reports exist on age-related changes occurring after the neonatal period.[31,32] In addition, the effect of BALF sample characteristics (i.e. BALF recovery, cell count, and cytological profile) has not been investigated in the horse. The purpose of our study was: 1) to measure surfactant

composition and function in healthy horses, and 2) to investigate the influence of age and BALF sample characteristics on surfactant variables.

Material and methods

Animals

Seventeen horses from the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) teaching herd were selected for this study. Horse breeds included 8 Thoroughbreds, 4 American Quarter Horses, 1 Arab, 1 Spanish Barb, and 3 mixed breed horses. Fourteen of the horses were mares and 3 horses were geldings. Ages ranged from 6 to 25 years of age (mean ± SD, 13 ± 6 years) and bodyweights ranged from 427 to 625 kg (mean ± SD, 538 ± 54 kg). Horses were housed on pasture year-around and had neither history nor clinical signs of respiratory disease for at least 1 year. All horses were routinely dewormed and vaccinated. During winter months, their diet was supplemented with hay. Criteria for enrollment in the study were normal parameters; of clinical examination, lung auscultation with rebreathing bag, and hematology, as well as absence of airway obstruction following exposure to barn environment. Samples were collected in June and July 2005. One day before sampling, horses were brought into the research barn where they were bedded on shavings and fed free-choice hay off the ground. Each horse had free access to a small individual outdoor pen. All procedures were approved by the Institutional Animal Care and Use Committee at the VMRCVM.

BALF collection and analysis

Horses were held in stocks and sedated by administration of detomidine hydrochloride[a] (0.01 mg/kg, IV) and butorphanol tartrate[b] (0.01 mg/kg, IV). A BALF collection tube[c] was passed through the nasal passages, into the trachea, and lower airways. Coughing was reduced by spraying the airways with a 0.2% lidocaine solution as the BALF collection tube was passed. Once the tube was wedged, BALF was collected by instilling 300 ml of prewarmed sterile saline solution and by reaspirating BALF with a syringe. Samples from each horse were pooled in a sterile specimen cup, mixed, placed on ice, and processed within 30 minutes after collection. Part of the sample was submitted for cell count and cytologic examination. The rest of the sample was centrifuged for 10 minutes at 400g and 4º C, the cell-free supernatant was stored at – 80º C until analysis.

BALF total cell counts were determined by using an automated cell counter[d]. For BALF

cytology, 300µl of each sample were applied to a slide by cytospin. Slides were stained by use of a quick Wright's stain. A 400-cell leukocyte differential count was performed on all slides.

Surfactant isolation and analysis

Cell-free BALF supernatant was centrifuged at 40,000 x g for 1 hour to allow separation of crude surfactant pellets (CSP) and surfactant supernatant (Supe). Supe was isolated and the CSP was washed two times with 100 ml of sterile saline (pH 7.4) and resuspended in a known volume of sterile saline.

Phospholipid content of CSP and Supe was measured following organic extraction[33] and by quantitation of lipid phosphorus.[34] Protein content of CSP and Supe was measured using the bicinchoninic acid method[e] with bovine serum albumin as the standard. Total phospholipid and protein content was calculated as the sum of their content in CSP and Supe. Percentages of phospholipid and protein content in CSP and Supe were calculated. A ratio was established between phospholipid content in CSP and Supe. Furthermore, the amount of protein per mg of phospholipid in CSP was evaluated.

Phospholipid composition of CSP was determined with high performance liquid chromatography (HPLC) equipped with an evaporative light scatter detector[f]. Previously described methods of gradient elution were used with a Kromasil silica column[g] as the solid phase.[17,27] A pulsating bubble surfactometer (PBS)[h] was used to evaluate surface tension lowering activity of CSP at a final concentration of 0.5 mg phospholipid/ml (in buffered saline containing 1.5mM $CaCl_2$).[35] Samples (40µl) were analyzed at 20 cycles/min for 10 minutes at 37ºC in the surfactometer.[17,27] Surface tension is reported as γ_{min}, which represents the minimum surface tension achieved over a 10 minute period.

Statistical analysis

Data are reported as mean, standard deviation, minimum, maximum, and 2.5 and 97.5 percentiles. Linear association between variables was estimated using correlation analysis with the CORR procedure of the SAS system[i].

Results

Clinical and BALF sample characteristics

Data for age, weight, BALF recovery, cell counts, and cell differentials are presented in table III.1. In one of the 17 horses, the BALF recovery percentage was only 42% and nucleated

cell count (NCC) was 2.8 x10^5/ml BALF. The sample from this horse was considered of insufficient quality for inclusion in the remaining data analysis. Data from another horse was excluded from the statistical analysis because it was an outlier in the data analysis for surfactant characteristics (see individual data provided below). The neutrophil percentage was less than 5% in BALF differential cell counts from eleven horses and was between 5 and 15% in the other four horses.

Surfactant composition and function

Surfactant variables from 15 horses are presented in table III.2. Surfactant surface tension from all the horses was <2mN/m. Surfactant variables from the horse with low BALF recovery percentage and low NCC mentioned above were: phospholipid content of CSP 36.75 µg/ml BALF, phospholipid content of Supe 2.27 µg/ml BALF, 91.67 % PC, 5.51 % PG, 1.09 % PI, 0.63 % PE, and 1.10 % SPH. Surfactant protein content was not determined from this sample. Another horse had a phospholipid content of CSP that was more than 3 standard deviations higher compared to the results from the rest of the horses. Data from this horse was therefore considered as outlying observations and was excluded from the statistical analysis. Surfactant characteristics from this horse were: phospholipid content of CSP 334.86 µg/ml BALF, phospholipid content of Supe 9.38 µg/ml BALF, protein content of CSP 16.40 µg/ml BALF, protein content of Supe 150.26 µg/ml BALF, 89.72 % PC, 8.65 % PG, 0.75 % PI, 0.51 % PE, and 0.36 % SPH.

Age and BALF sample characteristics relationships with surfactant variables

Correlation between age, weight, BALF characteristics and surfactant variables is presented in table III.3, while correlation amongst age, weight, and BALF characteristics is presented in table III.4. Increasing age was associated with decreased phospholipid content in CSP ($r = -0.6338$, $p = 0.0112$) but not Supe ($r = -0.3706$, $p = 0.1738$) (Figure III.1A and Table III.3). Age had no significant correlation with protein content in CSP ($r = -0.4928$, $p = 0.0620$) and Supe (-0.1146, $p = 0.6843$) (Figure III.1B) or phospholipid composition of surfactant (see table III.3). Age did not influence BALF recovery percentage ($r = -0.2486$, $p=0.3715$), nucleated cell count ($r = -0.1397$, $p = 0.6196$), or cell differentials (table III.4).

BALF recovery percentage and nucleated cell count were unrelated to phospholipid content in CSP and Supe, protein content in CSP and Supe, and phospholipid composition (table III.2). Neutrophil percentage of BALF was associated with decreasing percentage of SPH.

Removal or inclusion of the outlying observations from correlation analysis had in most cases only little influence on correlation coefficients. For example, the coefficient of determination for correlation between age and CSP varied from $r = 0.57$ to $r = 0.65$ depending on the inclusion/exclusion of outliers. However, for the correlation analysis between PG% and BALF recovery %, the correlation coefficient changed from $r = 0.43$ to $r = 0.63$ when data from the horse with low BALF recovery % and low NCC were included in the analysis.

Discussion

Our study demonstrated the presence of age-related surfactant changes in horses maintained on pasture. More specifically, older horses had lower phospholipid content in CSP, the surfactant-rich fraction of BALF. The age-related decrease in surfactant phospholipid was considered unaffected by variable sample recovery during BALF collection since neither BALF recovery percentage nor BALF nucleated cell count were associated with phospholipid content of surfactant. Low phospholipid content in CSP from older horses was unrelated to any compositional or functional changes of surfactant.

Surfactant changes are well described during pulmonary development in the neonate.[36] However, only a couple of articles exist in the literature and none using horses that report age-related surfactant changes in animals that are past the neonatal period.[31,32] Similar to our findings, evaluation of surfactant from 38 children without bronchopulmonary disease (ages 3 to 15 years-old), revealed an age-related decrease in surfactant phospholipid content (without changes in phospholipid composition).[31] A higher number of alveoli and smaller alveolar size were considered as possible explanations for higher surfactant content in young children. Increased proportion of PC and decreased proportion of SPH were reported in a study evaluating the effect of age and breed on surfactant composition in dogs.[32] The lack of information on the effect of age on surfactant composition may be explained by the fact that healthy subjects do not usually undergo diagnostic procedures such as BALF collection.

A number of changes occur in human respiratory function with age.[37,38] In humans, maximal pulmonary function is achieved at 20-25 years of age.[37] Ageing leads to a progressive

decrease in pulmonary performance and elastic recoil.[37,38] Decreased elastic recoil is associated with a loss of supporting tissue in peripheral airways due to a rearrangement of elastic fibers.[37,38] Alveolar surface area of the lung decreases with age because of an enlargement of airspaces.[37,38] If alveolar surface area also decreases with age in horses it may result in fewer numbers of type II alveolar cells or reduced surfactant production by these cells and therefore a decreased surfactant level in older horses. Information on age-related changes in pulmonary physiology and function in non-diseased older horses is limited. A reduction in the transfer of oxygen from alveoli to capillaries has been described in older horses.[39]

Our study had several limitations: a limited number of horses, absence of correction for variable recovery of BALF, and BALF neutrophilia in three of the horses. The majority of horses sampled were mares (13/15). Only a very small number of horses were evaluated for each breed. Due to these limitations it was not possible to evaluate an effect of breed or gender on surfactant.

The total phospholipid content in cell-free BALF was comparable to results obtained from healthy horses in other studies.[21,40] Phospholipid composition determined via HPLC in our study was characterized by slightly higher percentages of PC and slightly lower percentages of PG compared to results from other groups.[21,26,40] Surface tension determined from CSP was lower in our study in comparison to values reported by another group.[23] These differences in phospholipid composition and function may be explained by the use of different methodologies. In fact, each study on surfactant in horses has used different techniques to process and analyze samples.[21,26,40] Comparison of results between different studies should be done with caution and methods of sample processing and analysis should be reported.[1]

The measurement of dilutional factors (i.e. urea or albumin) has been proposed as a method to correct for the variable recovery of pulmonary epithelial lining fluid in BALF.[41-43] The use of dilutional factors is limited because concentrations of these factors are influenced by BALF dwell time and pulmonary permeability. According to guidelines from the European Respiratory Society results for acellular components of BALF should be expressed as quantities per milliliter of BALF recovered or as relative amounts.[44] Results from our study were expressed according to these guidelines and published reports on surfactant analysis in healthy and asthmatic human subjects.[17,45] Our analysis did not indicate any relationships between BALF

recovery percentage or NCC on surfactant characteristics at a BALF recovery percentage above 50%.

Healthy horses maintained on pasture have typically less than 5% neutrophils on BALF cytology.[46] Three of the horses sampled for our study had BALF cytology neutrophil percentages higher than 5% but lower than 15%. These increases in neutrophil percentages may be related to exposure of horses to hay since several reports exist in the literature on BALF neutrophilia occurring in healthy horses housed in a barn environment and fed hay.[47,48]

In conclusion, total surfactant phospholipid decreases with age in horses maintained on pasture. However, ageing does not influence surfactant phospholipid composition, or function. Further studies are needed to evaluate if decreased surfactant content in older horses correlate with changes in pulmonary function, histology, or responsiveness to a respiratory insult.

Manufacturers' addresses

[a] Detomidine, Pfizer, Exton, PA

[b] Butorphanol, Fort Dodge, Fort Dodge, IA

[c] Bivona, Gary, IN

[d] Casy R, Cell Size Analyzers, CELL Tools, Inc., San Francisco, CA

[e] Pierce, Rockford, IL

[f] SEDERE, Alfortville, France

[g] Drachrom, Greensboro, NC

[h] General Transco, Largo, FL

[i] the SAS system version 9.1.3 Service Pack 4, SAS institute Inc., Cary, NC

Table III.1: Table showing horses' (n=15) age and weight as well as BALF recovery percentage, BALF cell count, and cell differential. Data are presented as Mean ± SD.

Variable	Mean	SD
Age (years)	13.8	5.86
Weight (kg)	539	56.3
BALF Recovery (%)	68.5	7.0
NCC (x 10^5/ml BALF)	9.41	3.01
Neutrophil (%)	4.60	3.18
Macrophage (%)	52.53	12.13
Lymphocyte (%)	42.87	12.32

BALF: Bronchoalveolar lavage fluid, NCC: nucleated cell count.

Table III.2: Table of phospholipid, protein content, and phospholipid composition of BALF from 15 horses. Data are presented as mean, standard deviation, median, minimum, maximum, and 95% confidence interval.

Variable	Mean	SD	Median	Min	Max	95% CI
Phospholipid content						
PL Total (µg/ml)	143.4	59.0	143.1	36.1	242.8	110.7 – 176.1
PL CSP (µg/ml)	129	54.2	128.6	27.9	220.9	98.1 – 158.1
PL Supe (µg/ml)	15.3	7.05	13.6	7.25	31.5	11.4 – 19.2
CSP/Supe	8.79	2.82	9.13	3.41	12.4	7.2 – 10.3
Protein content						
Prot Total (µg/ml)	190	54.0	185.9	90.9	321.8	160.5- 220.3
Prot CSP (µg/ml)	9.51	4.70	8.8	2.6	17.0	6.91 – 12.1
Prot Supe (µg/ml)	181	52.5	172	87.6	313	151.8 – 209.9
Protein/Phospholipid (µg/ml/µg/ml)	1.66	1.09	1.2	0.6	4.6	1.1 – 2.2
Phospholipid composition						
PC (%)	90.6	0.95	90.7	88.5	92.6	90.1 – 91.2
PG (%)	7.45	0.75	7.35	6.25	9.18	7.01 – 7.86
PE (%)	0.57	0.21	0.61	0.0	0.8	0.53 – 0.69
PI (%)	0.91	0.14	0.93	0.6	1.07	0.84 – 0.99
SPH (%)	0.43	0.08	0.43	0.31	0.6	0.38 – 0.47
PC/PG	12.3	1.32	12.4	9.64	14.6	11.5 – 13.0

SD: standard deviation; Min: minimum; Max: maximum; 95% CI: 95 % confidence interval; PL Total: combined phospholipid content in CSP and Supe; PL CSP: phospholipid content in CSP, PL Supe: phospholipid content in supernatant; Prot Total: combined protein content in CSP and Supe; Prot CSP: protein content in CSP; Prot Supe: protein content in supernatant; Protein/Phospholipid: ratio of protein versus phospholipid content; PC: phosphatidylcholine; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; PI: phosphatidylinositol; SPH: sphingomyelin; PC/PG: ratio of PC versus PG.

*Note: Levels of PE and SPH were not detected on the chromatogram from one of the horses.

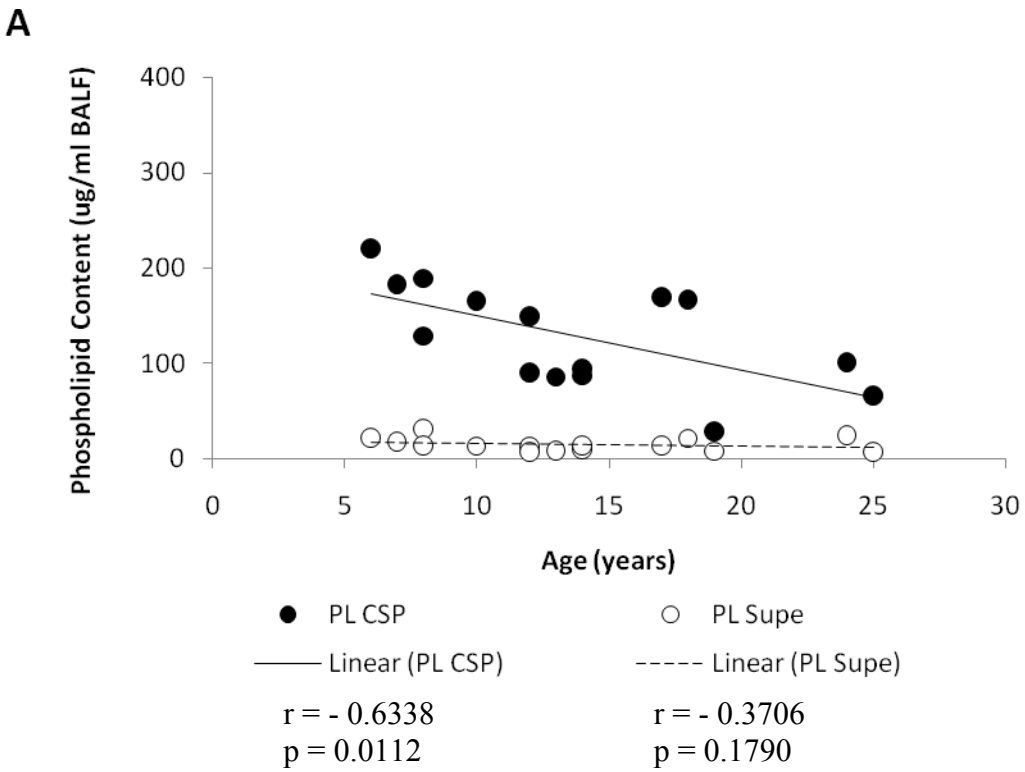
Table III.3: Correlation between age, weight, BALF characteristics and surfactant variables from healthy horses (n=15). Spearman correlation coefficients (r) and significance levels (p) are displayed. p-values < 0.05 are represented in bold characters.

		Age (Years)	**Weight** (kg)	**Recovery** (%)	**NCC** ($x10^5$/ml BALF)	**Neutrophil** (%)	**Lymphocyte** (%)	**Macrophage** (%)
PL Total	r	**-0.6303**	0.1683	0.3062	0.2714	0.0414	0.0804	-0.0090
(µg/ml BALF)	p	**0.0118**	0.5488	0.2671	0.3278	0.8834	0.7757	0.9747
PL CSP	r	**-0.6338**	0.1987	0.2632	0.2679	0.0180	0.0983	-0.0269
(µg/ml BALF)	p	**0.0112**	0.4776	0.3432	0.3344	0.9492	0.7274	0.9242
PL Supe	r	-0.3706	0.0286	0.1522	0.3214	0.0865	-0.2306	0.2655
(µg/ml BALF)	p	0.1738	0.9193	0.5882	0.2427	0.7592	0.4084	0.3389
Prot Total	r	-0.1790	-0.1110	0.3169	0.0964	0.4324	0.2180	-0.2547
(µg/ml BALF)	p	0.5232	0.6937	0.2498	0.7325	0.1074	0.4350	0.3596
Prot CSP	r	-0.4928	-0.0663	0.1201	0.3253	0.3499	0.0939	-0.1122
(µg/ml BALF)	p	0.0620	0.8144	0.6699	0.2368	0.2011	0.7392	0.6905
Prot Supe	r	-0.1146	-0.1002	0.2829	0.0321	0.4324	0.2466	-0.2906
(µg/ml BALF)	p	0.6843	0.7222	0.3069	0.9095	0.1074	0.3755	0.2934
PC	r	-0.0662	0.0161	-0.3223	-0.3357	-0.0595	-0.1216	0.1274
(%)	p	0.8145	0.9545	0.2414	0.2212	0.8333	0.6661	0.6510
PG	r	-0.0340	-0.0483	0.4297	0.2500	0.1910	-0.1144	0.1058
(%)	p	0.9042	0.8642	0.1099	0.3688	0.4953	0.6848	0.7074
PE	r	0.4088	-0.0486	0.2958	0.4295	-0.2870	0.4675	-0.3953
(%)	p	0.1467	0.8690	0.3045	0.1253	0.3198	0.0919	0.1618
PI	r	-0.2170	0.0359	-0.2430	0.0930	-0.0388	0.1262	-0.1438
(%)	p	0.4372	0.8990	0.3827	0.7416	0.8908	0.6540	0.6093
SPH	r	0.0652	0.4746	0.1004	-0.3304	**-0.6774**	0.4388	-0.2935
(%)	p	0.8248	0.0864	0.7326	0.2486	**0.0078**	0.1165	0.3085

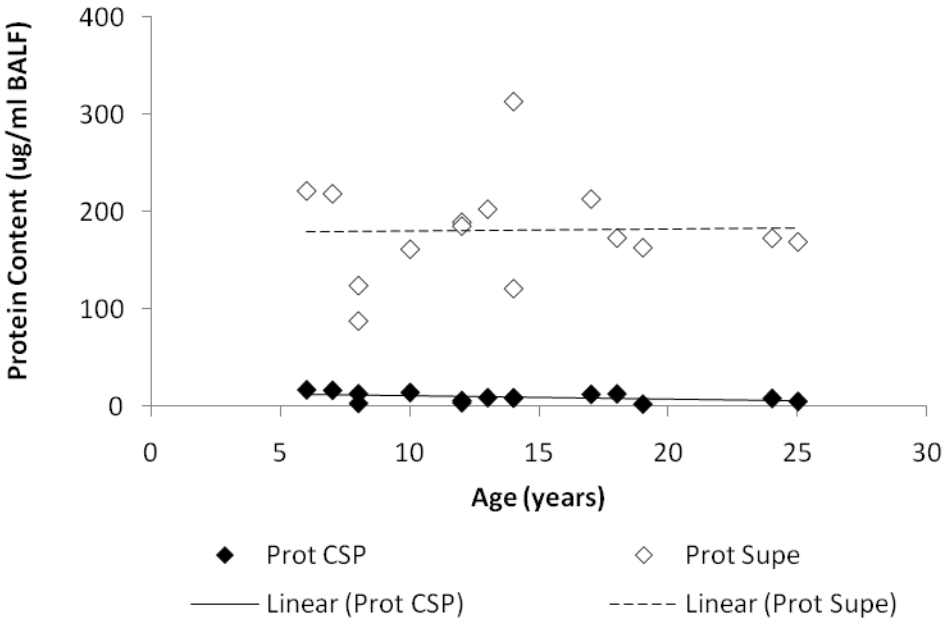
Table III.4: Correlation amongst age, weight, and BALF characteristics from healthy horses (n=15). Spearman correlation coefficients (r) and significance levels (p) are displayed. Correlations with a p-value < 0.05 are represented in bold characters.

		Age (Years)	**Weight** (Kg)	**% Recovery** (%)	**Cell/ml** ($x10^5$/ml BALF)	**Neutrophil** (%)	**Lymphocyte** (%)	**Macrophage** (%)
Age (Years)	r							
	p							
Weight (Kg)	r	-0.2253						
	p	0.4194						
Recovery (%)	r	-0.2486	-0.4120					
	p	0.3715	0.1270					
NCC ($x10^5$/ml BALF)	r	-0.1397	-0.1486	0.3563				
	p	0.6196	0.5971	0.1924				
Neutrophil (%)	r	-0.1635	**-0.5718**	0.2620	0.2306			
	p	0.5604	**0.0259**	0.3456	0.4082			
Lymphocyte (%)	r	0.2571	-0.2294	0.0367	-0.2109	-0.2218		
	p	0.3548	0.4108	0.8966	0.4505	0.4269		
Macrophage (%)	r	-0.3067	0.2815	0.0656	0.2547	0.0905	**-0.9722**	
	p	0.2663	0.3095	0.8162	0.3596	0.7484	**<0.0001**	

Figure III.1: Scatterplots and best-fit lines of phospholipid content (Panel A) and protein content (Panel B) in crude surfactant pellets (CSP) and supernatant (Supe) versus age in healthy horses (n=15). Spearman correlation coefficients (r) and corresponding significance levels are displayed.



B



r = - 0.4928
p = 0.0620

r = - 0.1146
p = 0.6843

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CHAPTER IV:

SURFACTANT ALTERATIONS IN HORSES WITH RECURRENT AIRWAY OBSTRUCTION (RAO) AT DIFFERENT CLINICAL STAGES

ABSTRACT

Objective: Examine surfactant phospholipid composition and function in RAO horses at different disease stages and compare to Non-RAO horses.

Animals: Seven horses with confirmed RAO and 7 Non-RAO horses.

Materials and Methods: Horses were evaluated in pairs (RAO/Non-RAO) while all horses were at baseline, during exposure to hay, and post-exposure. Evaluations included: clinical scoring, lung function testing, airway endoscopy, and bronchoalveolar lavage fluid (BALF) cell count and differential. Cell-free BALF was separated into Crude Surfactant Pellets (CSP) and supernatant (Supe) using ultracentrifugation and was analyzed for phospholipid and protein content. CSP phospholipid composition and surface tension were evaluated with HPLC and pulsating bubble surfactometer, respectively. Mixed effects repeated measures ANOVA was used for statistical analysis.

Results: Phospholipid content in cell-free BALF (CSP and Supe) was lower in RAO versus Non-RAO horses at all sample times. Ratio of BALF protein to phospholipid (TP/PL) was higher in RAO versus Non-RAO horses during Exposure compared to Baseline. In the RAO group, cell-free BALF phospholipid content was lower and TP/PL was higher during Exposure versus Baseline and Post-Exposure. No significant differences were found in BALF protein content, percentages of phospholipid classes, or surface tension of CSP between or within groups of horses.

Conclusions and Clinical Relevance: All clinical stages of RAO were characterized by low BALF phospholipid content. Clinical exacerbation led to further decrease in the phospholipid content and increased TP/PL ratio compared to Baseline. Causes for decreased surfactant in RAO horses remain to be determined. We speculate that low phospholipid content may render RAO horses more susceptible for surfactant alterations and contribute to clinical disease status and progression in these horses. Further study is warranted.

This article (Chapter IV) is planned to be submitted for publication to the American Journal of Veterinary Research (AJVR).

Introduction

Lung surfactant, produced by type II alveolar cells, lines the epithelial surface of the lung.[1-3] A large part (>80%) of surfactant is composed of phospholipids with a smaller portion of surfactant specific proteins (>10%), and neutral lipids (5-10%). Phospholipids confer surfactant its ability to lower surface tension (biophysical function). Surfactant proteins A and D participate primarily in innate immune defense mechanisms (immunologic function),[4,5] whereas surfactant proteins B and C contribute significantly to biophysical surfactant functions.[3]

Phospholipids are molecules with an amphipathic structure: a hydrophilic head region (determining phosopholipid class) and two hydrophobic tails (composed of fatty acid chains).[6] This molecular structure allows achievement of low surface tension and prevention of alveolar collapse.[1,2,6] Surfactant deficiency in premature animals leads to neonatal respiratory distress syndrome (NRDS).[7] NRDS is prevented and treated by administration of exogenous surfactant to premature infants.[3,7,8] Surfactant dysfunction (high surfactant surface tension) contributes to the pathophysiology of acute respiratory distress syndrome (ARDS).[9,10]

Airway surfactant, which is derived from alveolar surfactant, is essential to maintain airflow in small conducting airways.[11-14] Airway surfactant promotes mucociliary clearance, opposes airway edema, and reduces the sensitivity and overall magnitude of the bronchoconstrictive response.[15-18] Surfactant alterations have been described in a number of airway diseases, including human asthma.[2,11] Endobronchial allergen challenge in asthmatic patients induces surfactant dysfunction, serum protein infiltration, and alteration in the distribution of surfactant aggregates and phospholipids.[19-22] Protein inhibition is considered as one of the leading mechanisms of surfactant dysfunction in asthmatic patients.[19-21] Secretion of specific inflammatotory proteins such as eosinophilic cationic protein and hydrolysis of surfactant phospholipids via secretory phospholipase A2 further contribute to surfactant dysfunction.[22,23]

Surfactant alterations have previously been described in RAO horses with clinical disease exacerbation by our group and others.[24,25] Surfactant abnormalities included decrease in surfactant phospholipid content,[24] changes in surfactant aggregate ratio,[24] and in the ratio between phosphatidylcholine (PC) and phosphatidylglycerol (PG), the two main phospholipid

classes of surfactant.[24,25] Changes in surfactant composition and function that occur as a result of short term exposure to challenge environment have not been evaluated in RAO horses. The purpose of this study was to determine if surfactant alterations in RAO-affected horses change at different disease stages and if those changes correlate with disease severity.

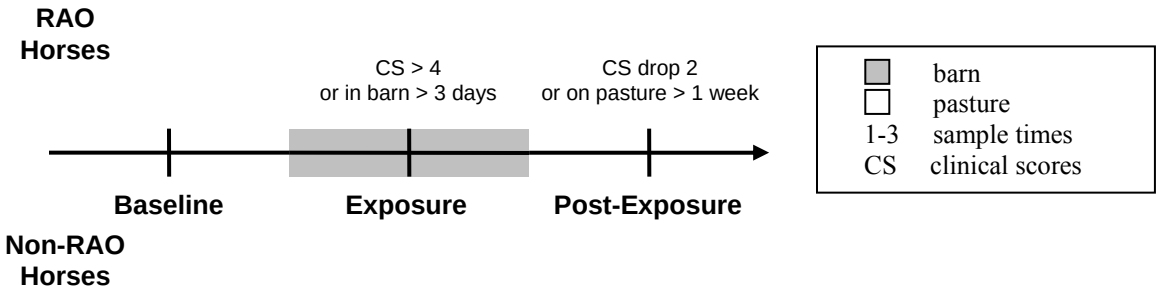
Materials and methods

Horses

Two groups of horses were used in the present study: Non-RAO horses and RAO horses. Non-RAO horses were part of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) equine teaching herd. These horses were housed on pasture all year long, had no history or clinical signs of respiratory disease for at least 1 year, and were known not to develop airway obstruction when housed in a barn and fed hay. RAO horses were part of a pre-existing herd of RAO horses (RAO herd) maintained at the VMRCVM. These horses were known to develop airway inflammation and obstruction when housed in a barn and fed moldy hay, and enter remission once pastured or stabled in a controlled environment.

Experimental protocol

Each RAO horse was paired with a Non-RAO horse and these horse pairs were subjected to the same sampling procedures and environmental changes in parallel. Whenever possible, horse pairs were of comparable age and weight (see Table IV.1.). Sampling times for each horse pair were determined by clinical stage in RAO horses.



Before the beginning of the study, all horses were maintained on pasture for a prolonged period of time (6-12 weeks) in order to optimize conditions for RAO horses to be in remission. The 'Baseline' sample was collected on horse pairs while RAO horses were in remission and had a clinical score (CS) of less than 4 according to the scoring system described by Robinson et al.[26] One week later, horses were brought into the research barn. Horses were housed in pairs in juxtaposed stalls to ensure similar environmental conditions. Horse pairs were bedded on dusty straw and fed dusty hay to create environmental challenge for the RAO horses. This environmental challenge was continued until RAO horses developed evident clinical signs of airway obstruction with a CS of more than 4 or had been in the barn for more than 3 days; the 'Exposure' sample was collected at this time. Horse pairs were then released back to pasture environment. The 'Post-Exposure' sample was collected when RAO horses were in recovery from airway obstruction as demonstrated by a decrease in CS by 2 points or following housing on pasture for more than one week.

Clinical scores, lung function testing, mucous scores (MS), and bronchoalveolar lavage fluid (BALF) collection.

Physical examination and CS were performed on each horse twice a day. At each sampling time a series of tests and procedures were performed and included: measurement of maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing, airway endoscopy with MS, and BALF collection and analysis.

Change in pleural pressure during tidal breathing was measured using techniques previously described.[26] Briefly, an esophageal balloon (10 cm long, 3.5 cm perimeter, 0.06 cm wall thickness) was sealed over the end of a polypropylene catheter (3 mm internal diameter, 4.4 mm external diameter). The tubing was passed into the distal third of the esophagus and attached to a very low range differential pressure transducer (Validyne Model DP/45-28)[a] calibrated before each study by means of a water manometer. The position of the esophageal balloon was adjusted to obtain the maximal changes in pleural pressure during tidal breathing (ΔPpl_{max}). Pleural pressure during breathing was obtained by a lung function computer.[b] At each data collection time, values of at least 15 consecutive breaths were averaged.

An endoscopic examination was performed on horses under sedation with butorphanol[c] (0.01 mg/kg) and detomidine[d] (0.01 mg/kg) given intravenously. The endoscope was passed through the nasal passages and into the trachea. The amount of mucus visible in the trachea was graded on a scale of 0 to 5 as previously described by Gerber at al.[27]

Bronchoalveolar lavage fluid (BALF) was collected using a cuffed BALF collection tube[e]. The tube was passed through the nasal passages and into the trachea. Lidocaine at 0.4% was infused to reduce coughing, the tube was advanced until wedged, and the cuff was inflated with 6 ml of air. Three aliquots of 100 ml (total volume = 300ml) of pre-warmed sterile saline solution were infused and reaspirated manually using a syringe. Aspirated fluid for each horse was mixed, pooled in a sterile specimen cup, and placed on ice. Recovered fluid volume and quality were recorded. Fluid samples were processed within 20 minutes after collection.

BALF fluid was centrifuged at 400 x g for 10 minutes. Cell-free supernatant was removed and further processed for isolation of surfactant (see below). Total nucleated cell count was determined with an automated cell counter[f]. Differential cell count was evaluated on slides prepared with cytospin[g] and stained with a modified Wrights stain. At least 400 cells in each specimen were counted under immersion microscopy.

Surfactant isolation and analysis

Cell-free BALF supernatant was centrifuged at 40,000 x g for 1 hour to allow its separation into 2 subfractions: crude surfactant pellet (CSP) and surfactant supernatant (Supe). Supe was isolated and the CSP was washed two times with 100 ml of normal saline (pH 7.4). CSP was then resuspended in a known volume of normal saline. Aliquots of Supe and CSP were stored at -80 °C.

Phospholipid content of CSP and Supe was measured following organic extraction[28] and by quantitation of lipid phosphorus.[29] Protein content of CSP and Supe was measured using the bicinchoninic acid method[h] with bovine serum albumin as the standard. Total phospholipid and protein contents were calculated as the sum of their respective contents in CSP and Supe.

In preparation for high performance liquid chromatography (HPLC), samples of CSP were extracted,[28] and the organic phase was dried under N_2, followed by resuspension in

chloroform. Phospholipid composition of CSP was determined with HPLC equipped with an evaporative light scatter detector[i]. Previously described methods of gradient elution were used with a Kromasil silica column[j] as the solid phase.[22,30] The amount of the following major phospholipid classes was determined: Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), and Sphingomyelin (SPM).

A pulsating bubble surfactometer (PBS)[k] was used to measure surface tension lowering activity of surfactant.[31] Surfactant from CSP was brought to a final concentration of 0.5 mg phospholipid/ml with buffered saline containing 1.5mM $CaCl_2$. Samples (40µl) were analyzed at 20 cycles/min for 10 min at 37 °C in the surfactometer as previously described.[32] Results were reported as γ_{min} which represents the minimum surface tension achieved over a 10 minute period.

In addition, commercial surfactant[l] was incubated with Supe from horses at 37ºC for 30 min to 4 hours. Following incubation, samples were prepared and evaluated as described above.

Statistical Data and Analysis

For all responses measured on a continuous scale a mixed effects, repeated measures analysis of variance (ANOVA) was used to test for main effects of disease and sample time as well as their interaction. The mixed procedure of the SAS[m] system was used: exposure status (RAO), sample time (Date), and their interaction (RAO*Date) were included in the model as fixed effects; horse pairs (Block) were included as random effects. Repeated measures (Date) were specified for horses within each horse pair and disease state (Horse (Block RAO)). The Tukey-Kramer adjustment was used for multiple comparisons. Data for cell counts, phospholipid and protein content was log-transformed for statistical analysis in order to fulfill model assumptions. Data in figures and tables is presented as either mean ± standard error or geometric mean and 95% confidence interval. Adjusted P-values <0.05 were considered as significant.

Exclusion criteria for samples

Initially, 11 horse pairs (Non-RAO/RAO) were included in the study and subjected to the 3 sample collections. Four horse pairs were excluded from the study due to insufficient sample recovery for analysis. Seven horse pairs were retained in the study.

Results

Clinical evaluation, lung function testing, mucous scores, and BALF analysis

In the Non-RAO group, no significant differences existed in CS between sample times. Non-RAO horses maintained CS below 4 at all sampling times (Figure IV.1, panel A). In the RAO group, CS were significantly different at each sample time. All RAO horses had CS below 4 at Baseline, CS increased to 5 or 6 (moderate disease) in 4 horses and to 7 or 8 (severe disease) in 3 horses during Exposure, while they reached intermediate levels during Post-Exposure. Furthermore, CS in RAO horses during Exposure and Post-Exposure were significantly higher compared to Non-RAO horses at any sample time.

Analysis of data from mucous scores (MS) revealed no significant interaction between the effect of RAO and sampling time. MS were significantly higher in RAO horses versus Non-RAO horses (all sample times combined). MS were significantly higher during Exposure and Post-Exposure versus Baseline (all horse groups combined). MS of Non-RAO horses were less than 1.5 at all sampling times (Figure IV.1, panel B), no significant differences existed between sample times. MS of RAO horses ranged from 1 to 3 at the different sampling times and were significantly higher during Post-Exposure versus Exposure.

For maximal pleural pressure changes (ΔPpl_{max}), a significant interaction existed between the effect of RAO and sampling time. In the Non-RAO group, no significant differences existed in ΔPpl_{max} between sample times. All Non-RAO horses had a ΔPpl_{max} of less than 8 cm H_2O at all sampling times (Figure IV.1, panel C). In RAO horses, ΔPpl_{max} was significantly higher during Exposure compared to Baseline. It was less than or equal to 8 cmH_2O at Baseline, higher than or equal to 20 cm H_2O in the majority of horses (n=6) during Exposure, and reached intermediate values during Post-Exposure. Values of ΔPpl_{max} only increased to 8 cmH_2O in one of the RAO horses during Exposure. Values of ΔPpl_{max} were significantly higher in RAO horses during Exposure compared to Non-RAO horses at any sample time.

Analysis of data from neutrophil percentage revealed no significant interaction between the effect of RAO and sampling time. Neutrophil percentage was significantly higher during Exposure compared to Baseline and Post-Exposure (all horse groups combined). In the Non-

RAO group, no significant differences existed in neutrophil percentage between sample times. Non-RAO horses had a BALF neutrophil percentage of less than 5% at Baseline (except in one horse that had a BALF neutrophil percentage of 25%), 10-25% during Exposure, and less than 10% during Post-Exposure (Figure IV.1, panel D). In the group of RAO horses, neutrophil percentage was significantly higher during Exposure compared to Baseline and Post-Exposure. Amongst the RAO horses, all horses had a neutrophil percentage of less than 10% at Baseline and during Post-Exposure (Table IV.2). Six of the RAO horses had a neutrophil percentage of more than 15% during Exposure. In one of the RAO horses, BALF neutrophil percentage reached only 11% during Exposure. Analysis of data from macrophage and lymphocyte percentage revealed no significant interaction between the effect of RAO and sample time. Macrophage percentage was significantly higher in Non-RAO horses versus RAO horses (all sampling times combined). Macrophage and lymphocyte percentages were significantly lower during Exposure compared to Baseline and Post-Exposure (all horse groups combined).

Analysis of data from BALF recovery percentages revealed no significant interaction between the effect of RAO and sampling time. Similarly, neither RAO nor sampling time had a significant effect on BALF recovery percentages (Table IV.2). For BALF cell counts, a near significant interaction existed between the effect of RAO and sample time. Cell counts were significantly lower during Post-Exposure in RAO horses versus Exposure and Post-Exposure in Non-RAO horses. No other significant differences existed.

Surfactant analysis

Phospholipid content (Total, CSP, and Supe)

For phospholipid content in cell-free BALF and its subfractions (CSP and Supe), no significant interactions existed between RAO and sample time. The effect of RAO was significant and phospholipid content in cell-free BALF and its subfractions (CSP and Supe) was significantly higher in Non-RAO horses compared to RAO horses (all sample times combined). The effect of sampling time was significant and phospholipid content in cell-free BALF and its subfractions was significantly higher at Baseline compared to Exposure (all horse groups combined). In addition, phospholipid content in Supe was significantly higher at Baseline compared to Post-Exposure (all horse groups combined).

More specifically, examination of differences in means revealed that phospholipid content in cell-free BALF and CSP was significantly higher at all sampling times in Non-RAO versus RAO horses (Figure IV.2, panel A) (Table IV.3). Phospholipid content in Supe was significantly higher in Non-RAO horses at Baseline compared to RAO horses during Exposure or Post-Exposure. In the group of RAO horses, cell-free BALF phospholipid content was higher at Baseline compared to Exposure.

Total protein content and total protein versus phospholipid ratio

Analysis of data from total protein content in BALF revealed no significant interaction between the effect of RAO and sample time. Total protein content in BALF was significantly higher at Baseline compared to Post-Exposure (all horse groups combined). No other significant differences were seen (Figure IV.2, panel B) (Table IV.3).

For the ratio between total protein and total phospholipid content in BALF (TPPL), a significant interaction existed between RAO and sample time. TPPL was significantly higher in RAO horses during Exposure compared to Non-RAO horses at all sample times (Figure IV.2, panel C). In the group of Non-RAO horses, TPPL stayed on average around 2 and did not change significantly over time. In the group of RAO horses, TPPL was on average between 5 and 6 at Baseline and Post-Exposure, whereas TPPL increased significantly during Exposure with an average above 9.

Phospholipid composition and surface tension

No significant interactions were found between RAO and sample time for the phospholipid composition as evaluated by percentage of each phospholipid class. The average phosphatidylglycerol (PG) content (% total) was lower in RAO versus Non-RAO horses (all sample times combined) but did not reach statistical significance (6.0 ± 0.2 versus 6.7 ± 0.2, $p=0.06$) (Table IV.3). Similarly, the average phosphatidylinositol content (% total) had a tendency to be higher in RAO horses versus Non-RAO horses (all sample times combined) (1.1 ± 0.1 versus 0.9 ± 0.1, $p=0.054$), but did not achieve statistical significance. Concentrations of PC, PG, and PI were significantly higher in Non-RAO horses versus RAO horses at all sample times, no interaction existed between RAO and sample time. No significant differences were

found in minimal surface tension between groups of RAO and Non-RAO horses or between sample times.

Discussion

Our study provides evidence that BALF phospholipid content is lower in RAO versus Non-RAO horses not only during clinical exacerbation (as previously reported)[24] but also in the absence of clinical signs. Exposure of RAO horses to challenge environment and ensuing clinical exacerbation lead to a further decrease in BALF phospholipid content and an increase in the ratio between BALF protein and phospholipid (TP/PL). In contrast to findings from our previous study[24] and the studies of others[25,33] no abnormalities were observed in surfactant phospholipid composition or function.

Causes of lung surfactant alterations include decreased surfactant synthesis and secretion by type 2 alveolar cells, increased surfactant degradation via inflammatory enzymes or cells, or inhibition of surfactant function through accumulation of edema fluid or inflammatory exudate.[2,11] Depending on the type and severity of inflammation present, several or all of these mechanisms may be present. In addition, it is possible that pulmonary disease influences the sampling of surfactant from the alveolar region.

Even though changes in surfactant aggregate ratio occur in asthmatic patients, an overall decrease of surfactant in those patients has not been described in the literature.[21,22] However, repeated allergen challenge in a murine model of asthma induced decreased surfactant levels and decreased PC synthesis.[34] Focal alveolar changes do occur in RAO horses and include alveolar hyperinflation and fibrosis.[35] Ultrastructural changes in type 2 alveolar cells from RAO horses included various degrees of cellular degeneration and accumulation of lamellar bodies (the cellular storage form of surfactant).[35] It is conceivable that these morphological changes in type 2 alveolar cells are accompanied by altered surfactant metabolism.[35] Evaluation of surfactant metabolism has been investigated in healthy human volunteers,[36] but has not been applied to horses.

Inflammatory enzymes such as phospholipases and proteases have the ability to degrade surfactant components.[6] In addition, macrophages and neutrophils show increased ability to

degrade surfactant during inflammation.[37] Our group and others previously reported decreased PG levels in RAO horses, indicating possible degradation of this specific surfactant component.[24,25] Similar findings had been reported in asthmatic patients with surfactant dysfunction.[22] Our present study only indicated a trend of lower PG levels in RAO horses. The number of horses in our study may have been too low to reveal a significant trend. It is also possible that a relatively short exposure to allergen and development of only moderate airway neytrophilia in RAO horses failed to induce changes in surfactant composition. On the other hand a more 'non specific' degradation of surfactant components by macrophages and neutrophils may have taken place in RAO horses.

Endobronchial allergen challenge leads to pronounced airway inflammation, surfactant dysfunction, and increased BALF protein concentration in human asthmatics.[19,21] A similar relationship between airway inflammation and surfactant dysfunction (as indicated by high surface tension) was not observed in our study. Specifically, no significant difference in surfactant function was observed between groups of horses or at different sampling times within groups of horses. In this study *in vitro* surfactant function was measured at a constant phospholipid concentration (0.5 mg/ml). Consequently, we cannot exclude an effect of diminished phospholipid content on the surface tension reducing ability of surfactant *in vivo*. Incubation of commercially available surfactant with sample supernatants (that typically contains the majority of inhibitory proteins) also did not provide evidence of surfactant inhibition by proteins in RAO horses (data not shown). These findings must be interpreted with caution since the proportion of protein versus phospholipid used *in vitro* likely does not mimic the ratio *in vivo.* Thus, the effect of a higher protein versus phospholipid ratio (TP/PL) observed in RAO horses in clinical exacerbation warrants further investigation.

Low phospholipid content in BALF and its subfractions persisted at all clinical stages in RAO horses whereas increased TP/PL ratio was characteristic of RAO following exposure to hay. Recently, RAO horses were shown to maintain a degree of airway obstruction (measured with forced expiratory flow) during remission.[38] These changes were believed to be related to persistent airway remodeling in RAO horses. RAO horses likely maintain a chronic level of airway inflammation during remission (even in the absence of evident BALF neutrophilia) similar to what has been described in asthmatic patients.[39] For example, increased levels of

myeloperoxidase (an enzyme released neutrophil respiratory burst) have been described in RAO horses 2 months following their return to pasture and without concurrent BALF neutrophilia.[40] Our findings provide evidence that surfactant alterations also persist during remission in RAO horses.

While all of the RAO horses in our study achieved clinical exacerbation as defined in the method section, airway neutrophilia was moderate in most horses and the period of time when horses demonstrated clinical compromise prior to sampling was brief (less than 48 hours). It is possible that changes in surfactant composition may be observed in horses with more severe airway neutrophilia. For example, endobronchial allergen challenge in asthmatic patients leads to pronounced airway inflammation and surfactant dysfunction whereas disease in non-challenged asthmatic patients does not induce a significantly increased BALF protein content or *in vitro* surface tension.[41]

The nature of our results (decreased phospholipid content in RAO horses without changes in phospholipid composition and function) may raise the question whether sample recovery from the bronchoalveolar region was less efficient in RAO compared to Non-RAO horses. For example, airway inflammation, bronchoconstriction, and excessive mucus accumulation in RAO may influence recovery of surfactant from the alveoli to the conducting airways. In addition, mucus accumulation may trap surfactant and prevent efficient recovery. However, percentage recovery was not significantly different between groups of horses and small differences seen were not enough to explain measured changes in phospholipid content. Measurement of dilutional markers has been suggested in order to evaluate dilution of pulmonary epithelial lining fluid (PELF) during BALF collection.[42,43] Concentrations of dilutional markers (i.e. urea or albumin) are influenced by dwell time, pulmonary permeability, and other factors and are therefore not considered reliable indicators of PELF dilution.[44] Current recommendations for the reporting of acellular components in BALF are to attempt standardization of BALF recovery volumes and report results in either amount per ml BALF recovered, or as a ratio or proportion of components.[44] In our study, results were reported according to these recommendations.

We speculate that low phospholipid content in BALF from RAO horses may render these horses more susceptible to surfactant alterations and dysfunction when airway inflammation

develops. In the absence of significant levels of surfactant inhibitors (i.e. protein) a substantial decrease in surfactant phospholipid content must occur to induce dysfunction.[45,46] *In vitro* experiments have demonstrated that surfactant maintains low surface tension at phospholipid concentrations as low as 0.3 mg/ml.[46] Surfactant in healthy animals is present in abundance which offers a protection against the effect of possible surfactant altering or inhibiting agents. Changes in surfactant phospholipid concentration can impact mucus wettability and mucociliary transport and may contribute to increased mucous accumulation in RAO horses.[15,47,48] Low surfactant levels may also affect bronchial hyperresponsiveness by two different mechanisms: unmasking of irritant receptor [49] and impaired smooth muscle relaxation.[16]

In conclusion, less surfactant is present in BALF from RAO horses at all clinical stages versus Non-RAO horses. Exposure of RAO horses to hay leads to decreased BALF phospholipid content and increased ratio between BALF protein and phospholipid. It is likely that low BALF phospholipid content in RAO horses is related to a combination of factors including changes in surfactant synthesis, secretion and/or passage to the conducting airways, surfactant degradation, and perhaps inhibition. Our study did not provide evidence for a specific cause of decreased surfactant levels nor of an injury to a specific surfactant component other than a possible reduction in PG content. Further studies, which will likely require a larger cohort of RAO horses, are needed to clarify these mechanisms. Furthermore, the influence of surfactant alterations on the pathophysiology of RAO remains unclear. We speculate that low surfactant phospholipid levels result from ongoing inflammation and remodeling in RAO horses and may contribute to the clinical manifestation of RAO including impaired mucus clearance and bronchial hyperresponsiveness.

Manufacturers' addresses

[a] Validyne Engineering Corp., Northridge, CA.

[b] Bucxo Electronics Inc., Wilmington, NC.

[c] Fort Dodge, Fort Dodge, IA.

[d] Pfizer, Exton, PA.

[e] Bivona, Gary, IN.

[f] Casy® Cell Size Analyzers, CELL tools Inc., San Francisco, CA.

[g] Thermo, Waltham, MA.

[h]Pierce, Rockford, IL.

[i] Sedere, Alfortville, France.

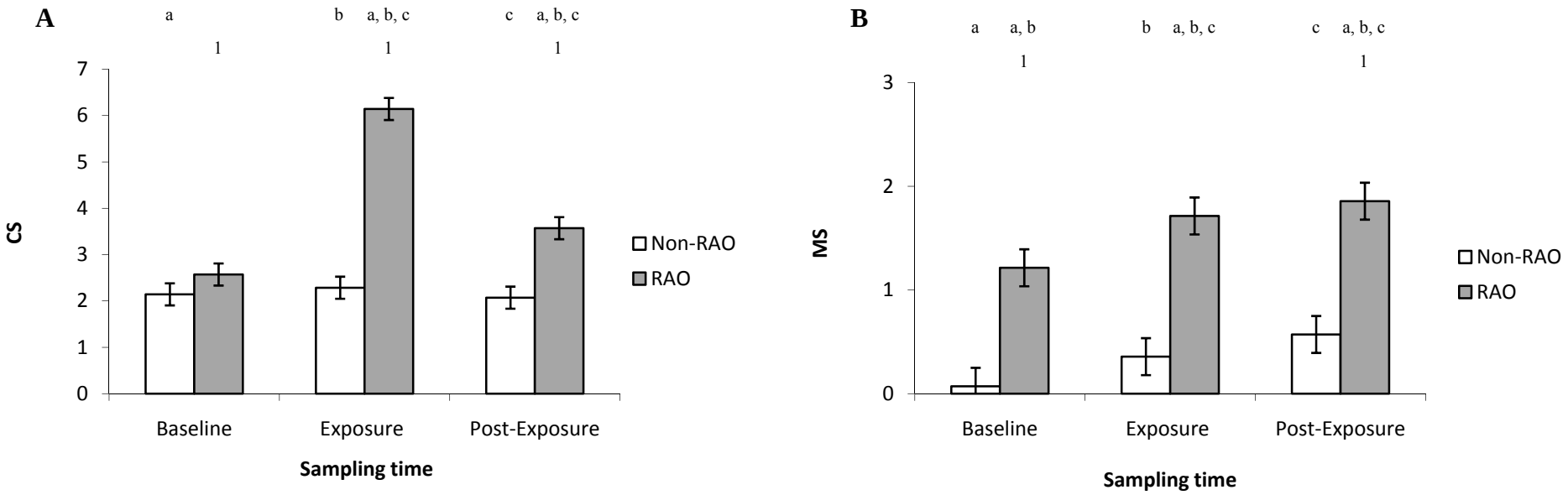
[j] Drachrom, Greensboro, NC.

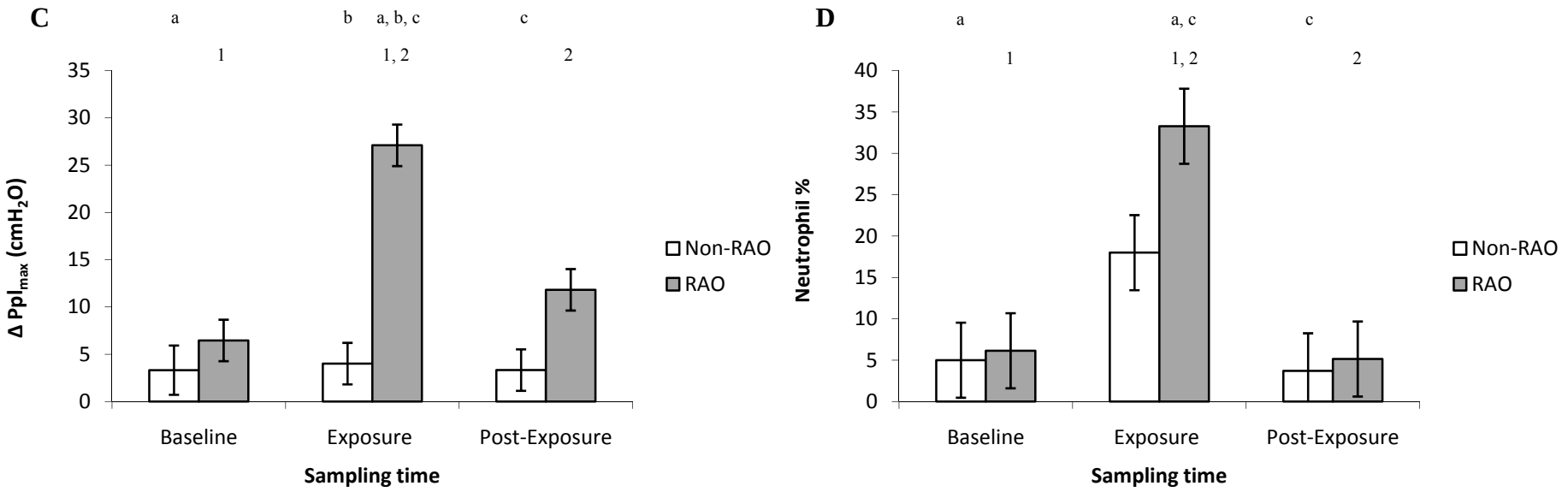
[k] General Transco, Largo, FL.

[l] Survanta ®, Abbott Nutrition, Columbus, OH

[m] The SAS system version 9.1.3. Service Pack 4, SAS Institute Inc., Cary, NC.

Figure IV.1: Graphical representation of clinical data. Data are presented for Non-RAO (n=7)* and RAO horses (n=7) at the different sampling times (Baseline, Exposure, and Post-Exposure). Panel A: Clinical scores (CS), Panel B: mucous scores (MS), Panel C: neutrophil percentage (Neutrophil %), and Panel D: maximal changes in pleural pressure (ΔPpl_{max}).





Data are presented as mean ± standard error.

[a] significant difference between Non-RAO horses at Baseline and RAO horses.

[b] significant difference between Non-RAO horses at Exposure and RAO horses.

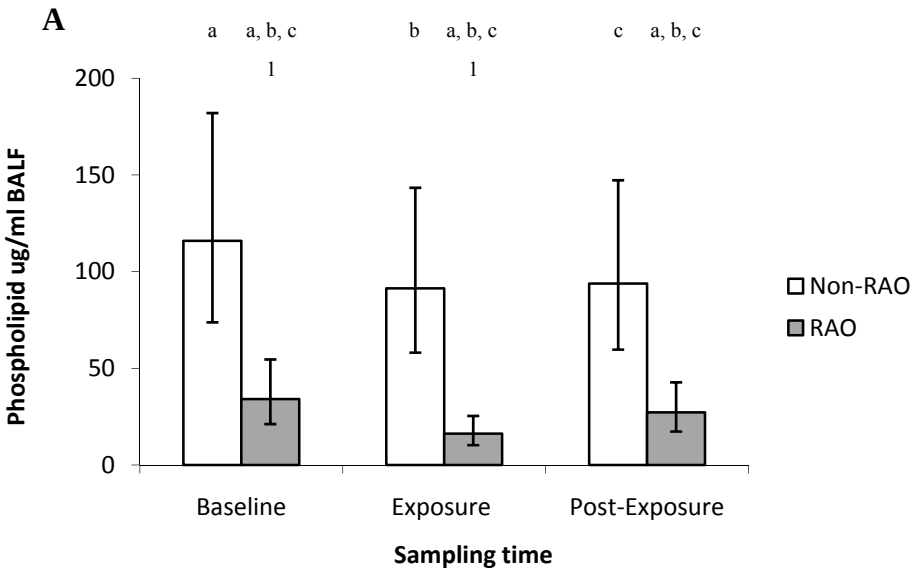
[c] significant difference between Non-RAO horses at Post-Exposure and RAO horses.

[1, 2] similar numbers represent significant differences within a group of horses.

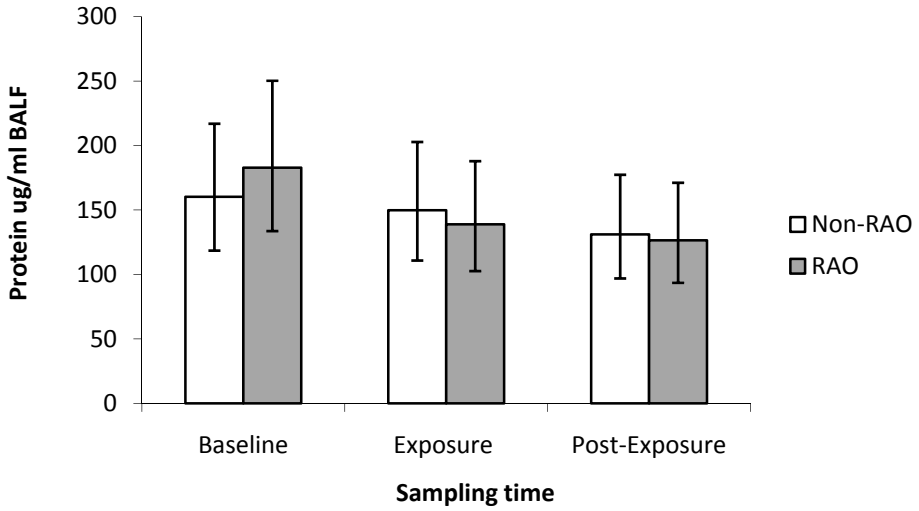
* ΔPpl_{max} data for Non-RAO horses at Baseline is only represented for n=5, since 2 horses were too uncooperative for measurement.

Note: lower limits of some of the error bars were truncated since none of the data presented can achieve negative values.

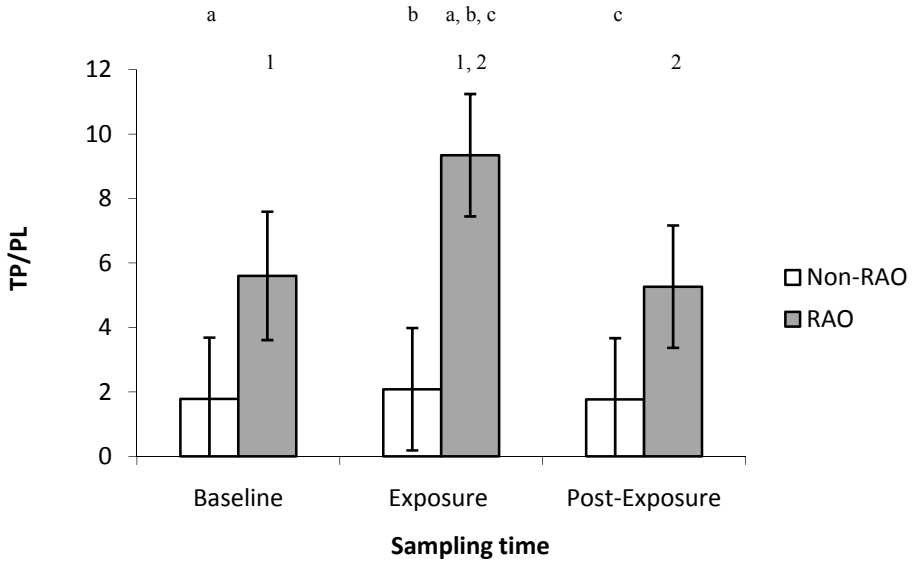
Figure IV.2: Graphical representation of phospholipid and protein content in cell-free BALF. Data are presented for Non-RAO (n=7) and RAO horses (n=7)* at the different sampling times (Baseline, Exposure, and Post-Exposure). Panel A: total phospholipid content, Panel B: total protein content, and Panel C: ratio between total protein and phospholipid content in cell-free BALF.



B


300
250
200
150
100
50
0
Protein ug/ml BALF
Baseline
Exposure
Post-Exposure
Sampling time
Non-RAO
RAO

C



Total phospholipid content and total protein content in cell-free BALF data are presented as geometric mean and 95% confidence interval since results were log-transformed for statistical analysis.

Ratio between total protein and phospholipid content (TPPL) data are presented as mean and standard error.

[a] significant difference between Non-RAO horses at Baseline and RAO horses.

[b] significant difference between Non-RAO horses at Exposure and RAO horses.

[c] significant difference between Non-RAO horses at Post-Exposure and RAO horses.

[1, 2] similar numbers represent significant differences within a group of horses.

* Data presented for RAO horses at Baseline is only represented for n=6, since samples from one horse were contaminated.

Note: lower limits of some of the error bars were truncated since none of the data presented can achieve negative values.

Table IV.1: Table showing individual horse data for the pairs of Non-RAO (n=7) and RAO horses (n=7). Pair number, age, sex, breed, and weight of each horse are displayed.

Non-RAO					RAO				
Pair	**Age (years)**	**Sex**	**Breed**	**Weight (kg)**	**Pair**	**Age (years)**	**Sex**	**Breed**	**Weight (kg)**
1	17	M	AQH	625	1	17	G	Pony	384
2	19	M	TB	560	2	19	G	TB	659
3	8	M	TB	560	3	18	M	AQH	477
4	10	G	AQH	498	4	12	M	TWH	449
5	18	M	TB	507	5	19	G	AQH	506
6	13	M	Arab	490	6	13	M	MB	500
7	14	M	App	600	7	20	M	TWH	530

M: mare, G: gelding, AQH: American Quarter Horse, TB: Thoroughbred, App: Appaloosa, TWH: Tennessee Walking Horse, MB: mixed breed.

Table IV.2: Table showing results for bronchoalveolar lavage fluid (BALF) recovery percentages, BALF cell count, and BALF cell differential. Data are presented for Non-RAO (n=7) and RAO horses (n=7) at the different sampling times (Baseline, Exposure, and Post-Exposure).

	Non-RAO			**RAO**		
	Baseline	Exposure	Post-Exposure	Baseline	Exposure	Post-Exposure
% BALF recovery	64.3 ± 4.7	59.4 ± 4.7	54.9 ± 4.7	50.9 ± 4.7	52.8 ± 4.7	57.7 ± 4.7
Cells x 10^5/ml	6.5 (4.8 – 8.8)	8.0 (5.9 – 10.8)[b]	8.4 (6.2 – 11.3)[c]	5.6 (4.1 – 7.6)	4.7 (3.5 – 6.4)	3.9 (2.9 – 5.3)[b, c]
Neutrophil %	5.0 ± 4.5[a]	18.0 ± 4.5	3.7 ± 4.5[c]	6.1 ± 4.5[1]	33.3 ± 4.5[a, c, 1, 2]	5.1 ± 4.5[2]
Macrophage %	45.4 ± 4.0	40.6 ± 4.0	49.7 ± 4.0[c]	41.3 ± 4.0	28.6 ± 4.0[c]	36.6 ± 4.0
Lymphocyte %	49.6 ± 4.7	41.0 ± 4.7	46.4 ± 4.7	52.6 ±4.7[1]	38.1 ± 4.7[1, 2]	58.7 ± 4.7[2]

Data fore % BALF recovery, Neutrophil %, Macrophage %, and Lymphocyte % are presented as mean ± standard error.

Data for BALF cell count (Cells x 10^5/ml) are presented as geometric mean and 95% confidence interval, since results were log-transformed for statistical analysis.

For the ratio between total protein and phospholipid content (TPPL) data are presented as mean and standard error.

[a] significant difference between Non-RAO horses at Baseline and RAO horses.

[b] significant difference between Non-RAO horses at Exposure and RAO horses.

[c] significant difference between Non-RAO horses at Post-Exposure and RAO horses.

[1, 2] similar numbers represent significant differences within the group of RAO horses.

Table IV.3: Table showing results of phospholipid content, protein content, and phospholipid composition. Data are presented for Non-RAO (n=7) and RAO horses (n=7)* at the different sampling times (Baseline, Exposure, and Post-Exposure).

	Non-RAO			RAO		
	Baseline	Exposure	Post-Exposure	Baseline	Exposure	Post-Exposure
Phospholipid content						
CSP (µg/ml BALF)	101.1 (61.1 – 167.4)[a]	82.5 (49.8 – 136.6)[b]	85.1 (51.4 – 140.9) [c]	22.8 (13.5 – 38.6)[a, b, c]	12.3 (7.4 – 20.4)[a, b, c]	22.2 (13.4 – 36.7)[a, b, c]
Supe (µg/ml BALF)	13.3 (8.4 – 21.0)[a]	8.0 (5.1 – 12.7)	7.8 (4.9 – 12.4)	8.6 (5.4 – 13.6)	3.5 (2.2 – 5.5)[a]	4.7 (3.0 – 7.4)[a]
Protein content						
Total (µg/ml BALF)	160.3 (118.4 – 216.9)	149.8 (110.7 – 202.8)	131.0 (96.8 – 177.3)	182.8 (133.6 – 250.2)	138.8 (102.6 – 187.9)	126.4 (93.4 – 171.1)
Supe (µg/ml BALF)	150.7 (110.3 – 205.8)	140.8 (103.1 – 192.3)	122.9 (90.0 – 167.8)	180.1 (130.3 – 248.9)	137.2 (100.5 – 197.4)	124.4 (91.1 – 169.9)
Phospholipid composition						
PC (%)	91.2 ± 0.3	91.1 ± 0.3	92.0 ± 0.3	91.8 ± 0.3	91.7 ± 0.3	91.6 ± 0.3
PG (%)	7.0 ± 0.2	6.8 ± 0.2	6.3 ± 0.2	6.1 ± 0.3	6.0 ± 0.2	6.2 ± 0.2
PE (%)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
PI (%)	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
SPH (%)	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.0

Total: cell-free BALF; CSP: crude surfactant pellet; Supe: surfactant supernatant; PC: phosphatidylcholine; PG : phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanolamine; SPH: sphingomyelin.

Data for phospholipid and protein content are presented as geometric mean and 95% confidence interval, since results were log-transformed for statistical analysis.

Data for phospholipid composition are presented as mean ± standard error.

For the ratio between total protein and phospholipid content (TPPL) data are presented as mean and standard error.

[a] significant difference between Non-RAO horses at Baseline and RAO horses.

[b] significant difference between Non-RAO horses at Exposure and RAO horses.

[c] significant difference between Non-RAO horses at Post-Exposure and RAO horses.

[1, 2] similar numbers represent significant differences within the group of RAO horses.

* Data presented for RAO horses at Baseline is only represented for n=6, since samples from one horse were contaminated.

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CHAPTER V:

ARE CLINICAL STAGES OF RECURRENT AIRWAY OBSTRUCTION RELATED TO SURFACTANT ABNORMALITIES?

ABSTRACT

Objective: Evaluate possible relationships between surfactant alterations and RAO characteristics.

Animals: Seven horses with confirmed RAO and 7 Non-RAO horses.

Materials and Methods: Horses were evaluated in pairs (RAO/Non-RAO) while RAO horses were at baseline, during exposure to hay, and post-exposure. Evaluations included: clinical scores (CS), maximal change in pleural pressure during tidal breathing (ΔPpl_{max}), endoscopic determination of airway mucous scores (MS), and bronchoalveolar lavage fluid (BALF) cell count and differential. BALF was separated into crude surfactant pellets (CSP) and supernatant (Supe) using ultracentrifugation. Phospholipid and protein content were determined from both of these subfractions, using organic extraction with determination of total phosphorus and bicinchoninic acid method respectively. CSP phospholipid composition and surface tension were evaluated with HPLC and pulsating bubble surfactometer, respectively. Correlation analysis was used including all horse groups and sample times. In addition, correlations were evaluated separately according to group and sample time.

Results: A positive correlation existed between Supe protein content and BALF neutrophil count and percentage in RAO horses during Exposure (r=0.9) as well as overall ΔPpl_{max} (r=0.6). A weak negative correlation was found between clinical scores and percentage phosphatidylglycerol (PG) (r=0.4).

Conclusions and Clinical Relevance: Airway neutrophilia and ΔPpl_{max} did not correlate with surfactant phospholipid content and composition. The relationship between airway neutrophilia, ΔPpl_{max}, and protein content was likely due to serum protein infiltration or increase in locally produced protein. Future studies should include evaluation specific protein composition. In addition, surfactant should be evaluated from RAO horses following prolonged remission and in association with methods that assess subclinical airway obstruction, airway hyperreactivity, or structural pulmonary changes.

This article (Chapter V) is planned to be submitted for publication to the American Journal of Veterinary Research (AJVR).

Introduction

Lung surfactant is composed of phospholipids and a proportionally smaller content of surfactant specific protein, and neutral lipids. Surfactant is produced in alveolar type 2 cells but also lines the rest of the epithelial lung surface. Airway surfactant promotes patency of small conducting airways,[1,2] improves muco-ciliary clearance,[3,4] and reduces severity of bronchoconstriction to inhaled allergens.[5-7] Surfactant alterations are believed to exacerbate disease severity in airway diseases, as seen in human asthma.[8-10] Allergen challenge in asthmatic patients induces surfactant dysfunction (increased surface tension).[11,12] Abnormalities in surfactant composition in these patients include increased protein content in broncholaveolar lavage fluid (BALF),[11,12] changes in the surfactant aggregate ratio,[12,13] and changes in either phosphatidylcholine (PC)[14] or phosphatidylglycerol (PG) levels.[13]

Information on the relationship between disease severity and surfactant abnormalities is relatively limited.[15,16] Studies involving asthmatic patients subjected to endobronchial allergen challenge include patients with severe airway inflammation.[11-13] Two studies described changes in surfactant in asthmatic patients without allergen challenge.[15,16] The first study evaluated sputum from asthmatic patients (stable and in crisis) and healthy patients.[16] Surface tension in asthmatic patients negatively correlated with their forced expiratory volume in one second (FEV1), providing evidence that high surface tension is related to airway obstruction. The second study evaluated surfactant composition and function in BALF and sputum from asthmatic patients.[15] No significant changes were found in percentages of the main phospholipid classes; however a weak relationship existed between FEV1 and the percentage of dipalmitoyl-PC (the main phospholipid molecular species of surfactant).

We have focused on phospholipid analysis of surfactant since these components are largely responsible for its biophysical properties. We previously reported decreased surfactant (phospholipid) levels in horses with clinical disease exacerbation of RAO.[17] In another study (see Chapter IV), we evaluated surfactant in RAO horses at different clinical stages: in remission (at baseline), with clinical disease exacerbation following exposure to hay, and post-exposure with improvement of clinical signs. Phospholipid content in cell-free BALF decreased from baseline to expsure. Furthermore, the ratio between protein and phospholipid content in BALF was

significantly higher in RAO horses in crisis versus remission and recovery. The purpose of the present study was to evaluate possible correlations between clinical stage of RAO and surfactant variables.

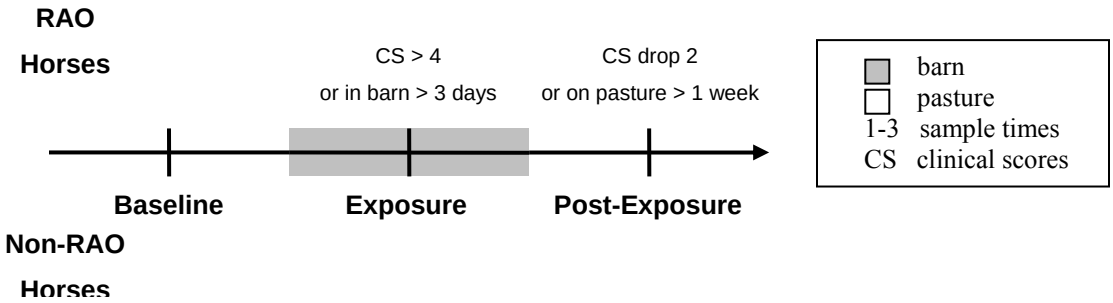
Material and methods

Horses

Two groups of horses were used in the present study: Non-RAO horses and RAO horses. Non-RAO horses were part of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) equine teaching herd. These horses were housed on pasture all year long, had no history or clinical signs of respiratory disease for at least 1 year, and were known not to develop airway obstruction when housed in a barn and fed hay. RAO horses were part of a pre-existing herd of RAO horses (RAO herd) maintained at the VMRCVM. These horses were known to develop airway inflammation and obstruction when housed in a barn and fed moldy hay, and enter remission once pastured or stabled in a controlled environment.

Experimental protocol

Each RAO horse was paired with a Non-RAO horse and these horse pairs were subjected to the same sampling procedures and environmental changes in parallel. Whenever possible, horse pairs were of comparable age and weight (see Table IV.1., Chapter IV). Sampling times for each horse pair were determined by disease stage in RAO horses.



Before the beginning of the study, all horses were maintained on pasture for a prolonged period of time (6-12 weeks) in order to optimize conditions for RAO horses to be in remission. The 'Baseline' sample was collected on horse pairs while RAO horses were in remission and had a clinical score (CS) of less than 4 according to the scoring system described by Robinson et al.[18] One week later, horses were brought into the research barn. Horses were housed in pairs in juxtaposed stalls to ensure similar environmental conditions. Horse pairs were bedded on dusty straw and fed dusty hay to create environmental challenge for the RAO horses. This environmental challenge was continued until RAO horses developed evident clinical signs of airway obstruction with a CS of more than 4 or had been in the barn for more than 3 days; the 'Exposure' sample was collected at this time. Horse pairs were then released back to pasture environment. The 'Post-Exposure' sample was collected when RAO horses were in recovery from airway obstruction as demonstrated by a decrease in CS by 2 points or following housing on pasture for more than one week.

Clinical scores, lung function testing, mucous scores (MS), and bronchoalveolar lavage fluid (BALF) collection.

Physical examination and CS were performed on each horse twice a day. At each sampling time a series of tests and procedures were performed and included: measurement of maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing, airway endoscopy with MS, and BALF collection and analysis.

Change in pleural pressure during tidal breathing was measured using techniques previously described. Briefly, an esophageal balloon (10 cm long, 3.5 cm perimeter, 0.06 cm wall thickness) was sealed over the end of a polypropylene catheter (3 mm internal diameter, 4.4 mm external diameter). The tubing was passed into the distal third of the esophagus and attached to a very low range differential pressure transducer (Validyne Model DP/45-28)[a] calibrated before each study by means of a water manometer. The position of the esophageal balloon was adjusted to obtain the maximal changes in pleural pressure during tidal breathing (ΔPpl_{max}). Pleural pressure during breathing was obtained by a lung function computer.[b] At each data collection time, values of at least 15 consecutive breaths were averaged.

An endoscopic examination was performed on horses under sedation with butorphanol[c] (0.01 mg/kg) and detomidine[d] (0.01 mg/kg) given intravenously. The endoscope was passed through the nasal passages and into the trachea. The amount of mucus visible in the trachea was graded on a scale of 0 to 5 as previously described by Gerber at al.[19]

Bronchoalveolar lavage fluid (BALF) was collected using a cuffed BALF collection tube[e]. The tube was passed through the nasal passages and into the trachea. Lidocaine at 0.4% was infused to reduce coughing, the tube was advanced until wedged, and the cuff was inflated with 6 ml of air. Three aliquots of 100 ml (total volume = 300ml) of pre-warmed sterile saline solution were infused and reaspirated manually using a syringe. Aspirated fluid for each horse was mixed, pooled in a sterile specimen cup, and placed on ice. Recovered fluid volume and quality were recorded. Fluid samples were processed within 20 minutes after collection.

BALF fluid was centrifuged at 400 x g for 10 minutes. Cell-free supernatant was removed and further processed for isolation of surfactant (see below). Total nucleated cell count was determined with an automated cell counter[f]. Differential cell count was evaluated on slides prepared with cytospin[g] and stained with a modified Wrights stain. At least 400 cells in each specimen were counted under immersion microscopy.

Surfactant isolation and analysis

Cell-free BALF supernatant was centrifuged at 40,000 x g for 1 hour to allow its separation into 2 subfractions: crude surfactant pellet (CSP) and surfactant supernatant (Supe). Supe was isolated and the CSP was washed two times with 100 ml of normal saline (pH 7.4). CSP was then resuspended in a known volume of normal saline. Aliquots of Supe and CSP were stored at -80 °C.

Phospholipid content of CSP and Supe was measured following organic extraction[20] and by quantitation of lipid phosphorus.[21] Protein content of CSP and Supe was measured using the bicinchoninic acid method[h] with bovine serum albumin as the standard. Total phospholipid and protein contents were calculated as the sum of their respective contents in CSP and Supe.

In preparation for high performance liquid chromatography (HPLC), samples of CSP were extracted,[20] and the organic phase was dried under N_2, followed by resuspension in chloroform. Phospholipid composition of CSP was determined with HPLC equipped with an evaporative light scatter detector[i]. Previously described methods of gradient elution were used with a Kromasil silica column[j] as the solid phase.[13,22] The amount of the following major phospholipid classes was determined: Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), and Sphingomyelin (SPM).

A pulsating bubble surfactometer (PBS)[k] was used to measure surface tension lowering activity of surfactant.[23] Surfactant from CSP was brought to a final concentration of 0.5 mg phospholipid/ml with buffered saline containing 1.5mM $CaCl_2$. Samples (40µl) were analyzed at 20 cycles/min for 10 min at 37 °C in the surfactometer, as previously described.[24] Results were reported as γ_{min} which represents the minimum surface tension achieved over a 10 minute period.

In addition, commercial surfactant[l] was incubated with Supe from horses at 37ºC for 30 min to 4 hours. Following incubation, samples were prepared and evaluated as described above.

Statistical Data and Analysis

Correlation analysis was performed between variables that characterize clinical stage of RAO (CS, MS, ΔPpl_{max}, neutrophil numbers and percentage in BALF) and selected surfactant variables. The latter included: phospholipid content in CSP (surfactant-rich fraction of cell-free BALF), protein content in Supe (containing the majority of soluble proteins), PC and PG (the 2 major phospholipid classes in surfactant). Correlation analysis was performed separately for groups of horses (RAO and Non-RAO) at each sample time.

In addition, correlations between clinical variables and surfactant variables were estimated using PROC MIXED in SAS. A model was formulated according to the method described by Hamlett et al.,[25] which allows assessment of correlation in the presence of repeated measures. The model included treatment (RAO) and time (Date) effects as well as their interaction. Block (RAO and Non-RAO horse pairs) was included as a random effect while horse was used as a subject effect to define the repeated measurements (Date). The objective of this analysis was to explore the relationships between clinical and surfactant variables, therefore we

report the correlation coefficients as a correlation matrix without assessment of statistical significance.

For all responses measured on a continuous scale a mixed effects, repeated measures analysis of variance (ANOVA) was used to test for main effects of disease and sample time as well as their interaction. The mixed procedure of the SAS^{m} system was used: exposure status (RAO), sample time (Date), and their interaction (RAO*Date) were included in the model as fixed effects; horse pairs (Block) were included as random effects. Repeated measures (Date) were specified for horses within each horse pair and disease state (Horse (Block RAO)). The Tukey-Kramer adjustment was used for multiple comparisons. Data in figures and tables is presented as either mean ± standard error or geometric mean and 95% confidence interval (see Chapter IV). Adjusted P-values <0.05 were considered as significant.

Exclusion criteria for samples

Initially, 11 horse pairs (Non-RAO/RAO) were included in the study and subjected to the 3 sample collections. Four horse pairs were excluded from the study due to insufficient sample recovery for analysis. Seven horse pairs were retained in the study.

Results

Clinical variables and surfactant variables

Detailed results for clinical and surfactant variables are described in the previous chapter (Figure IV.1 and IV.2). A brief summary of these results is re-stated here.

In the group of Non-RAO horses CS, MS, ΔPpl_{max} did not change over time. However, the relative and absolute amount of neutrophils was increased during Exposure of Non-RAO horses to hay. In the group of RAO horses, CS, MS, ΔPpl_{max}, absolute and relative amounts of neutrophils increased significantly during exposure and reached intermediate levels Post-Exposure.

Phospholipid content in cell-free BALF was significantly lower during Exposure compared to Baseline in RAO horses. The ratio between total protein and phospholipid (TP/PL)

was significantly higher in RAO horses during Exposure compared to Baseline and Post-Exposure.

Correlation analysis between clinical and surfactant variables

Correlation analysis including both Non-RAO and RAO horse groups and the 3 sampling times (Baseline, Exposure, and Post-Exposure) is presented in table V.1. A weak negative correlation existed between CS and PG percentage ($r = -0.378$, $p = NA$), and a moderate positive correlation existed between protein content in Supe and ΔPpl_{max} ($r=0.644$, $p = NA$). P-values were not available for this analysis (see material and methods section of this chapter).

A breakdown of simple correlations between airway neutrophilia, ΔPpl_{max}, and selected surfactant variables within each group of horses and at each sampling time is presented in table V.2. No significant correlations were found between these variables in Non-RAO horses. In RAO horses, a significant positive correlation existed between the amount and percentage of neutrophils and protein content in Supe ($r = 0.928$, $p = 0.0025$ and $r = 0.900$, $p = 0.0056$) (see Figure V.1).

Discussion

In the present study we found that a higher supernatant protein content was strongly associated with airway neutrophilia in RAO horses. Furthermore, higher protein content was also related to increased airway obstruction. Horses with most clinical signs of airway obstruction tended to have lower surfactant phosphatidylglycerol (PG) levels.

The majority of BALF protein (>90%) is contained in the supernatant (Supe) fraction which, in our study, was analyzed separately from the 'phospholipid rich' fraction of BALF (CSP). This means that the protein content that we refer to herein should be considered as a BALF characteristic rather than a surfactant variable. Protein recovered in BALF is either plasma protein diffused through the alveolo-capillary barrier or is produced locally by different cell types.[26] Airway inflammation typically leads to an increase in both of these protein categories but can also induce a depletion in certain locally produced proteins.[27] A more detailed protein analysis was not performed in our study, since its primary objective was to evaluate phospholipid

alteration in surfactant. Future studies should include this analysis to determine if the relationship between BALF protein and neutrophils is due to non-specific extravasation of serum protein or a more specific locally produced protein.

Higher BALF protein levels were associated with more severe airway obstruction in our study. Measurable airway obstruction did not develop in Non-RAO horses following exposure to hay and subsequent airway neutrophilia in several horses. Furthermore, airway neutrophilia secondary to exposure to barn environment has been described in Non-RAO in several studies.[28,29] Causes for airway inflammation in Non-RAO horses are still poorly understood. It has been proposed that airway inflammation in RAO horses is associated with an increased neutrophil activation state,[28] an upregulation in nuclear factor kappa-B (NF-κB),[30] delayed neutrophil apoptosis,[31] and increased levels of myeloperoxidase during recovery from crisis.[32] The fact that RAO horses develop signs of airway obstruction with airway neutrophilia following exposure to barn environment may be related to persistent airway remodeling present in RAO horses. A recent study that used forced expiratory flow to evaluate respiratory compromise in RAO horses showed that subclinical airway obstruction was present even in remission.[33] At this stage, RAO horses did not show clinical signs of RAO and classic lung function testing did not reveal airway obstruction.

Despite our findings that RAO horses had a lower BALF phospholipid content during Exposure compared to Baseline (see previous chapter), no significant correlation was found between surfactant levels and clinical signs, ΔPpl_{max}, or airway neutrophilia. Several explanations are possible for these findings. Firstly, we previously found that surfactant levels are lower in RAO horses versus Non-RAO horses even at Baseline, in the absence of clinical signs, airway obstruction (as measured by ΔPpl_{max}), or airway neutrophilia. A correlation between low surfactant levels and these variables was therefore unlikely. Future studies should include more sensitive assessments of airway obstruction such as evaluation of airway hyperreactivity or remodeling and might yield more positive findings. Compliance rather than ΔPpl_{max} would have been a more interesting parameter to assess a relationship between surfactant alterations and airway obstruction. Nevertheless, classic lung function testing is in general not sensitive enough to detect subclinical airway obstruction.[18,33] Secondly, airway neutrophilia was moderate in a number of RAO horses during Exposure. While low surfactant

levels may predispose RAO horses for further surfactant alterations and functional consequences, a more severe and/or chronic airway inflammation than that observed in our study may be necessary to show a possible relationship to airway obstruction and inflammation. Lastly, it would also be very interesting to assess RAO horses that only recently developed the disease, for the first time in their lives. This would allow to assess them potentially with minimum of underlying airway remodeling.

A study in asthmatic patients showed evidence that severity of airway obstruction was related to high surfactant surface tension.[16] Studies that evaluated asthmatic patients following endobronchial allergen challenge showed correlations between surfactant surface tension and percentage of BALF eosinophils or protein content.[12,13] However, detailed data on other possible relationships between clinical data and surfactant variables is not available. In our study, a trend existed between decreased surfactant phosphatidylglycerol (PG) percentage and higher clinical scores. This finding supports our observation that PG percentage tends to be lower in surfactant from RAO versus Non-RAO horses (see previous chapter). It is also strengthened by our previous studies that demonstrated decreased PG in RAO horses.[17] It is conceivable that PG is more sensitive to degradation by inflammatory enzymes liberated during the disease process of RAO.

In conclusion, airway neutrophilia and ΔPpl_{max} did not correlate with surfactant phospholipid content and composition. The relationship between airway neutrophilia, ΔPpl_{max}, and protein content was likely due to serum protein infiltration or increase in locally produced protein. More severe clinical signs of RAO were weakly associated with lower PG. Future studies should include evaluation specific protein composition. In addition, surfactant should be evaluated from RAO horses following prolonged remission and in association with methods that assess subclinical airway obstruction, airway hyperreactivity, or structural pulmonary changes.

Manufacturers' addresses

[a] Validyne engineering Corp., Northridge, CA.

[b] Bucxo Electronics Inc., Wilmington, NC.

[c] Fort Dodge, Fort Dodge, IA.

[d] Pfizer, Exton, PA.

[e] Bivona, Gary, IN.

[f] Casy® Cell Size Analyzers, CELL tools Inc., San Francisco, CA.

[g] Thermo, Waltham, MA.

[h]Pierce, Rockford, IL.

[i] Sedere, Alfortville, France.

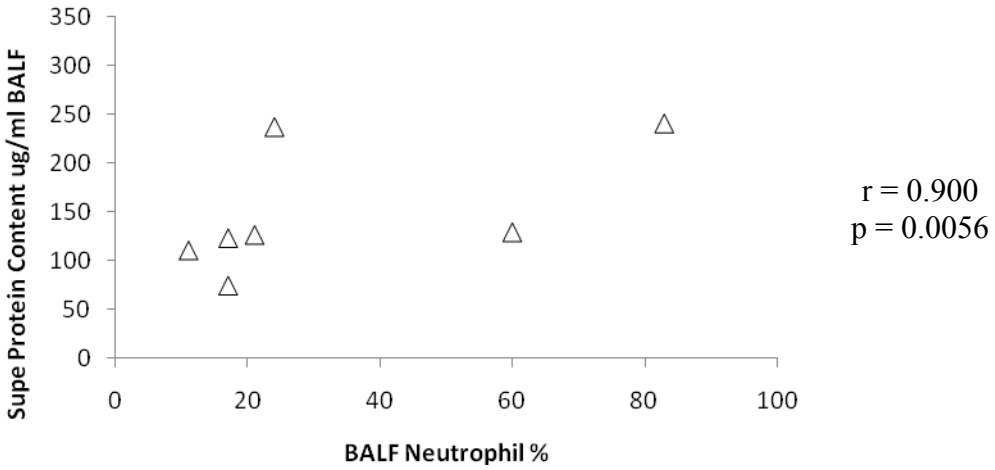
[j] Drachrom, Greensboro, NC.

[k] General Transco, Largo, FL.

[l] Survanta ®, Abbott Nutrition, Columbus, OH

[m] The SAS system version 9.1.3. Service Pack 4, SAS Institute Inc., Cary, NC.

Figure V.1.: Scatter plot of Bronchoalveolar lavage fluid (BALF) neutrophil percentage versus supernatant (Supe) protein content in the group of RAO horses during crisis Exposure. Spearman correlation coefficient (r) and significance level (p) are displayed.



r = 0.900
p = 0.0056

Table V.1: Correlation between clinical variables and surfactant variables including all horse groups (Non-RAO and RAO) and sampling times. Correlation analysis was performed according to the method described by Hamlett et al[25] which allow assessment of correlation in the presence of repeated measures. This method explored relationships between variables but did not assess statistical significance. The numbers displayed are correlation coefficients (r).

	Surfactant variables			
Clinical Variables	PL CSP (µg/ml BALF)	Prot Supe (µg/ml BALF)	PC (%)	PG (%)
CS	-0.042	0.220	-0.262	-0.378
MS	-0.0316	0.226	-0.099	-0.091
Neutro ($x10^5$/ml)	0.350	0.106	0.197	0.238
ΔPpl_{max} (cmH_2O)	0.239	0.644	NA	NA

CS: clinical score, MS: mucous score, Neutro: neutrophil percentage of BALF, ΔPpl_{max}: maximal pleural pressure change, PL CSP: phospholipid content in crude surfactant pellets, Prot Supe: protein content in supernatant, PC: phosphatidylcholine, PG: phosphatidylglycerol, NA: not applicable.

Table V.2: Correlation between selected surfactant variables, BALF neutrophil counts (Neutro/ml), and maximal pleural pressure changes (ΔPpl_{max}) for each group of horses (Non-RAO and RAO) and the different sampling times (Baseline, Exposure, and Post-Exposure). Results are indicated as Spearman correlation coefficients (r) and significance levels (p).

Non-RAO															
		Baseline					**Exposure**					**Post-Exposure**			
		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)
Neutro/ml	r	0.321	0.143	-0.714	0.357	r	-0.286	0.607	-0.357	0.357		-0.25	0.643	0.214	-0.714
($x10^5$/ml BALF)	p	0.482	0.76	0.07	0.432	p	0.534	0.148	0.432	0.432		0.589	0.119	0.644	0.071
ΔPpl_{max}	r	0.4	-0.6	0.2	-0.5	r	0.286	0.107	0.143	0.107		-0.342	-0.324	-0.882	0.234
(cmH_2O)	p	0.505	0.285	0.747	0.391	p	0.534	0.819	0.76	0.819		0.452	0.478	0.008	0.613
RAO															
		Baseline					**Exposure**					**Post-Exposure**			
		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)		PL CSP (µg/mlBALF)	Prot Supe (µg/mlBALF)	PC (%)	PG (%)
Neutro/ml	r	0.257	-0.371	-0.643	0.75	r	0.393	**0.928**	0.321	0.393		0.571	0.464	0.071	0.286
($x10^5$/ml BALF)	p	0.623	0.468	0.119	0.052	p	0.383	**0.002**	0.482	0.383		0.18	0.294	0.879	0.534
ΔPpl_{max}	r	-0.714	0.086	0.071	-0.25	r	-0.162	-0.324	0.09	-0.216		0.071	0.143	0.536	-0.393
(cmH_2O)	p	0.111	0.871	0.879	0.589	p	0.728	0.475	0.848	0.641		0.879	0.76	0.215	0.383

PL CSP: phospholipid content in crude surfactant pellets, Prot Supe: protein content in supernatant, PC: phosphatidylcholine, PG: phosphatidylglycerol.

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SUMMARY AND CONCLUSIONS

Surfactant alterations are well described in airway diseases in human medicine but are still poorly characterized in the horse. The first study presented in this thesis (CHAPTER III) evaluated surfactant composition and function in healthy horses on pasture. In addition, possible influences of horse age and BALF sample characteristics on surfactant composition were assessed. We established a basis for typical surfactant composition and function in healthy horses. Furthermore, we found that phospholipid content in the 'phospholipid rich' fraction of BALF (CSP), but not its supernatant (Supe), progressively decreased with age. BALF sample characteristics such as recovery percentage and cell count did not influence surfactant (phospholipid) levels, composition, or function. Our findings were consistent with some the available published literature on this subject. A number of age related changes occur in respiratory function in human medicine. Therefore, a more detailed evaluation of pulmonary function in older horses including correlation between structural and functional changes to their surfactant composition would likely yield interesting observations.

Thus far, changes in surfactant composition had only been described in RAO horses during clinical exacerbation. Our studies presented in CHAPTER IV and V were the first to evaluate surfactant changes in RAO horses at different clinical stages. Briefly, our objectives were 1) to examine surfactant phospholipid composition and function in RAO horses at different clinical stages (at baseline, following exposure to hay, and post-exposure) and compare to Non-RAO horses (CHAPTER IV), and 2) to evaluate possible relationships between surfactant alterations and disease severity (CHAPTER V).

In CHAPTER IV we provided evidence that low surfactant (phospholipid) levels are present in RAO versus Non-RAO horses at different clinical stages: at baseline (without clinical signs), following exposure to hay (clinical exacerbation), and post-exposure (improvement in clinical signs). In RAO horses, exposure to hay induced a further decrease in surfactant content and a relative increase of protein versus phospholipid in BALF. Phospholipid composition and function did not change significantly. Results from the study described in CHAPTER V revealed that higher BALF protein content was related to airway neutrophilia in RAO horses and overall more pronounced airway obstruction. A weak association was also found between higher clinical

scores and lower phosphatidylglycerol. No significant correlation was evident between surfactant phospholipid content and airway neutrophilia or maximal changes in pleural pressure during tidal breathing.

Possible consequences of decreased surfactant in the bronchioli of RAO horses include: impaired muco-ciliary clearance (by changes in mucus viscoelasticity and transport), increased airway hyperreactivity (by decreased barrier against stimulation from inhaled allergens), and more pronounced airway inflammation (by upregulation of inflammatory cytokines), all of which lead to an exacerbation in airway obstruction. In our study (CHAPTER IV and V), low surfactant levels in RAO at baseline were not accompanied by clinical signs, airway obstruction (as measured in our study), or airway neutrophilia. Future studies should therefore aim to investigate relationships between surfactant variables and measures of subclinical airway obstruction (e.g. forced expiratory flow), airway remodeling (e.g histology of lung biopsy specimen), and/or hyperreactivity in RAO horses during remission (e.g. histamine bronchoprovocation). It is conceivable that low surfactant levels in RAO horses are an indicator of ongoing airway obstruction, remodeling, and potentially inflammation in RAO horses during remission.

Causes of surfactant alterations and function in airway disease include: inhibition of surfactant function by protein infiltration, surfactant degradation by inflammatory enzymes or cells, and altered surfactant synthesis and secretion. Evidence from research in human asthma and animal asthma models indicates that many of these events may take place during airway inflammation. We observed an increase in the ratio of protein versus phospholipid in RAO horses during crisis and found a correlation between BALF protein levels and airway inflammation and obstruction. However, surfactant dysfunction or inhibition was not evident in our samples. Decreased phosphatidylglycerol levels in RAO horses may be an indicator that surfactant degradation occurs in these horses. Finally, focal changes in type 2 alveolar cells have previously been described in RAO horses and were suggested as a cause for altered surfactant metabolism. Evaluation of lung histology (e.g. biopsy or postmortem) in parallel with surfactant analysis is necessary to prove this theory. In addition, evaluation of lung surfactant from pulmonary tissue could also exclude effects of BALF sample procedure on surfactant.

Our study did have several limitations: small number of horses, mild to moderate airway neutrophilia in RAO horses, methods used to evaluate lung function in horses, and analysis of surfactant function. Even though we consistently found low phospholipid content in RAO horses, a higher number of samples would strengthened our conclusions. Induction of a stronger airway neutrophilia in RAO horses (by prolonged exposure to hay) could have lead to additional changes in surfactant phospholipid composition and perhaps function. Alternatively, evaluation of RAO horses following prolonged remission or of horses that only recently developed clinical signs of RAO could have elucidated if low surfactant levels in RAO horses were due to progressive airway remodeling that occurs with persistent subclinical disease. In retrospect, since surfactant levels were low in RAO horses at baseline (when all parameters of classic lung function testing may be normal) inclusion of forced expiratory mechanics (not available at our facilities) or histamine bronchoprovocation would have been beneficial in our study. Functional analysis of surfactant with a methodology that more closely mimics conditions in lower airways (versus alveoli) may provide greater sensitivity for identifying surfactant dysfunction.

In conclusion, our study showed that low surfactant levels are present in RAO horses at baseline (remission) and are worsened following exposure to hay. It is conceivable that a low amount of bronchiolar surfactant in RAO horses may contribute their propensity to develop airway obstruction, mucous accumulation, and bronchial hyperresponsiveness. This may be exacerbated during exposure to hay when a relatively higher protein versus phospholipid ratio is present. Furthermore, a progressive decrease of surfactant levels in older horses may contribute to a worsening of clinical signs in older RAO-affected horses.

Several interesting directions exist for future research. It would be valuable to assess surfactant levels in RAO remission in relation to airway hyperreactivity following bronchoprovocation and to velocity of tracheal mucociliary clearance. Furthermore, it will be interesting to assess a large number of RAO horses with variable disease duration and severity to clarify if surfactant alterations develop secondary to progressive airway remodeling in RAO. Finally, analysis of pulmonary surfactant metabolism, which has recently become available in human medicine, may represent a new way to assess surfactant synthesis and secretion in the horse.

APPENDIX

ANALYTICAL METHODS

Surfactant Isolation

1. Centrifuge cell-free BALF at 40,000 x g for 1 hr + 5 min, +4°C.
2. Carefully collect Supernatanat (Supe) with pipette and transfer to labeled 5 ml cryovials, store at -80°C.
3. Resuspend pellets (CSP) with sterile normal saline.
4. Centrifuge at 40,000 x g for 1 hr + 5 min, +4°C.
5. Repeat steps 3-4.
6. Suspend CSP in a small, known volume of saline.
7. Store aliquot surfactant pellet suspension in cryovials at -80°C.

Sample extraction

1. Prepare triplicates for each sample (CSP or Supe).
2. To each tube add:
   - For pellet 5 µl surfactant and 495 µl saline
   - For supernatant 1000 µl supernatant
3. Add 1 volume MeOH
4. Vortex
5. Add 1 volume $CHCl_3$
6. Add 1 volume of MeOH
7. Vortex
8. Add 2 volumes $CHCL_3$
9. Add 1 volume dH_2O
10. Vortex
11. Centrifuge at 500-600 g for 4 min
12. Aspirate bottom layer and place into new glass tube
13. Add 2 volumes $CHCl_3$ to remaining top layer
14. Vortex top layer
15. Recentrifuge (see step 11)

16. Aspirate bottom layer and add pool with previously collected aspirate (see step 12)

Drying

Dry samples under a continuous stream of Nitrogen gas.

Quantitation of total phosphorus

Prepare standards in triplicates of KH_2PO_4 (0.025 to 5mM).

1. Add 20 µl of saline to blank and 20 ul of dH_2O to dried lipid samples. Add 20 µl of standard to respective test tubes
2. Add 150 µl of 70-80% perchloric acid to each tube and vortex
3. Incubate tubes in heat block at 170-190°C for 25 minutes
4. Allow tubes to cool to room temperature
5. Add 900 µl of sterile H_2O to each tube and vortex.
6. Add 167 µl of 2.5% ammonium molybdate to each tube and vortex
7. Add 167 µl of 10% ascorbic acid to each tube and vortex
8. Incubate in 50°C water bath for 15 min
9. Allow tubes to cool to room temperature
10. Place 1000 µl of each tube into a Sartstedt cuvette
11. Measure absorbances of samples and standards at 820 nm wavelength using cuvette reader.

HPLC analysis

Phospholipid composition of CSP was determined by separation of individual phospholipids with high performance liquid chromatography (HPLC) using a Kromasil silica column (Drachrom; Greensboro, NC) and a dual solvent system gradient from 100% Mobile Phase A ($CHCl_3$:MeOH:NH_4OH; 80:19.5:0.5) ramped to a maximum of 25% Mobile Phase B (MeOH:H_2O:NH_4OH; 80:19.5:0.5). Quantitation of individual phospholipid peaks from the HPLC eluent was performed with an evaporative light scatter detector (SEDERE; Alfortville, France). Retention times and binomial response characteristics of individual phospholipids and lysophospholipids were defined by use of commercially available standards (Avanti Polar Lipids, Alabaster, AL). This sensitive method measured individual phospholipids in the range of 0.1 to

4.0 nmoles and required a minimum surfactant sample size of 12 nmoles. To calculate absolute phospholipid concentrations, a fixed concentration of phosphatidylbutanol (PB) was added to a known concentration of sample after lipid extraction in order to serve as an internal standard.

The amount of the following major phospholipid classes was determined: Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Sphingomyelin (SPH).

Surface activity evaluation with the pulsating bubble surfactometer (PBS)

A pulsating bubble surfactometer (PBS) (General Transco; Seminole, FL) was used to measure surface tension lowering activity. Surfactant from the CSP fraction (40µl) was used at a final concentration of 0.5 mg phospholipid/ml in Tris buffered saline (pH 7.4) containing 1.5mM $CaCl_2$, and was analyzed at 20 cycles/min for 10 min at 37°C in the surfactometer. Results were reported as γ_{min}, which represented as the minimum surface tension achieved over the 10 minute period.