NATURE AND CAUSES OF MUCUS ACCUMULATION IN EQUINE LOWER AIRWAY DISEASE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Large Animal Clinical Sciences

2003

ABSTRACT

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The purpose of my work was to quantify airway mucus accumulation and investigate its causes in RAO-affected horses and clinically healthy controls. I hypothesized that mucus accumulation is a function of the interaction of disease status (RAO), environment (dust exposure) and age of the horse. My investigations showed that endoscopic scoring is a reliable tool to quantify mucus accumulation. Using endoscopic scoring to compare RAO-affected and clinically healthy horses, I found very large mucus accumulations to be limited to RAO-affected horses, especially during acute exacerbation. There was less mucus accumulation in controls, and I found no difference between age groups. Variation between individuals was high, however, with many clinically healthy horses showing signs of IAD. Overall, mucus accumulation was associated with neutrophilic airway inflammation. I then proposed that increased accumulation is a consequence of unfavorable rheological changes and/or increased mucin production. I found that increases of mucus accumulation in RAO exacerbation are accompanied by a large rise of mucus viscoelasticity, which in turn is at least partly due to F-actin fibers, likely released from degenerate neutrophils. These rheological changes could not explain the increased mucus accumulation in RAO remission or the overall high variability, however. Therefore, I identified equine homologues of gel-forming

mucin genes. EqMUC5AC, but not eqMUC2 showed robust expression in large and small airway generations of both RAO-affected and clinically healthy horses. A preliminary semi-quantitative study on pooled samples suggested that eqMUC5AC expression is increased in RAO. I identified equine homologues of hCACC1 and EGFR, key signaling elements in mucin gene regulation, and developed quantitative RT-PCR assays for eqMUC5AC, equine hCACC1-like, EGFR and EGF. This allowed me to accurately determine mRNA levels in individual samples and investigate the association of eqMUC5AC expression with the expression of these key signaling elements, with stored intraepithelial mucosubstance and with neutrophilic inflammation. My findings did not support the hypothesis that eqMUC5AC is up-regulated and intraepithelial mucosubstances are increased in RAO-affected compared to clinically healthy control horses. EqMUC5AC, equine hCACC1-like, EGFR or EGF mRNA levels showed remarkable consistency across different lung lobes within individuals, indicating that the underlying pathogenesis is a diffuse process. Conversely, differences were considerable between horses. Equine hCACC1-like and EGFR mRNA levels as well as neutrophil percentages were associated with eqMUC5AC mRNA levels in both groups. In conclusion, gross airway mucus accumulation is increased in RAO-affected horses, particularly during exacerbation. The increase during exacerbation can partly be explained by unfavorable rheological changes. The roles and pathogenesis of increased mucin production and mucus cell hyperplasia in RAO remain unresolved. My findings indicate, however, that EqMUC5AC, equine hCACC1-like, EGFR and neutrophilic inflammation may be involved in mucus production in the airways of horses suffering from RAO as well as of horses with milder degrees of airway inflammation.

Dedicated to my father, Heinz Gerber

ACKNOWLEDGMENTS

I wish to express my gratitude to my major professor, Dr. N. E. Robinson, the best mentor one could wish for, and my thanks to my guidance committee, Drs. F. Derksen, J. Hotchkiss, J. Harkema and H. Schott. Many others have given me crucial help and advice in specific areas: Drs. S. Ewart, P. Gehr, J. Hauptman, V. ImHof, M. King, M. Kiupel, E. Marti, P. Venta, D. Schneider, R. Straub and J. Wagner. Absolutely central to the success of my thesis work was the help of Cathy Berney and Heather deFeijter-Rupp. I would also like to acknowledge the assistance of D. Aebischer, L. Bartner, G. Bleuer, D. Boruta, E. Curat, U. Du Pasquier, S. Eberhart, B. Feller, A. Jefcoat, J. Klumpp, A. Lindberg, M. Ricklin , J. Rawson and F. Uehlinger. As an "outsider", my brother Manuel Gerber has given me very helpful advice on many manuscripts, and the help of Victoria Hoelzer-Maddox has been invaluable preparing manuscripts for submission and in meeting the laughably excessive and largely pointless formatting requirements of this thesis. Faith Peterson has helped me navigate graduate school throughout my PhD candidate years. My fiancée Shannon Axiak deserves my thanks for her understanding and support when I wasn't always the most pleasant to be with during all that work. I also thank my mother for her untiring help and support across the Atlantic. Finally, I would like to acknowledge the funding agencies of USDA Animal Health and Well-Being; the Matilda Wilson Endowment, 3M, USA and Novartis, Switzerland that have helped fund this research. I am especially indebted to the Swiss National Science Foundation for the fellowship that allowed me to come to Michigan State University five years ago, and thus started it all.

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INTRODUCTION

In European classical times, respiration was thought to be a cooling mechanism for the blood, and nasal mucus a discharge from the brain. While Arabic medicine already possessed a much clearer understanding of physiology in medieval times, the credit of pointing out the pathophysiological significance of respiratory secretions to Western medicine belongs to Laennec. Excess mucous accumulation became one of the cardinal signs and a recognized problem of many respiratory diseases of humans.

The airway mucus apparatus (i.e., mucous secretory cells, ciliated cells, and progenitor cells) is a component of the innate mucosal defense system, which can respond rapidly to inhaled toxicants, microbial pathogens, and particulate matter by increasing mucus production and clearance in order to trap and remove the offending agents. If production and secretion exceed clearance capacity, however, excessive mucus accumulation is observed in the airways.

Excess mucus accumulation in the airways is a hallmark of equine recurrent airway obstruction (RAO) [1-3]. When exposed to conventional stable environment, i.e., hay feeding and bedding on straw, RAO-affected horses develop overt respiratory distress, i.e., heaves. In these severely affected animals the presence of large quantities of thick sticky-appearing mucus in large and small pulmonary airways is a common observation. Bronchoscopy, however, has expanded the recognition of excessive mucus accumulation to a wider range of horses. In particular, mucus accumulation has become a hallmark of inflammatory airway disease (IAD), a milder form of airway disease that is more difficult to define.

Three major pathophysiological components of equine lower airway disease are recognized: mucus accumulation, airway inflammation and bronchospasm/airway hyperreactivity. Compared with the latter two components, the nature and causes of mucus accumulation have received much less investigative attention and are therefore less well understood. In the following literature review, I examine the available information on mucus accumulation in equine lower airway diseases with a focus on RAO and IAD. Comparative aspects are included when relevant to develop the conceptual model and resulting hypotheses that form the basis of my PhD work:

The focus of my investigations is the excess mucus accumulation that occurs in equine RAO. In order to determine if this excess airway mucus is caused either by increased production and/or by decreased clearance, I did the following:

- validated an endoscopic scoring system and used it to test the hypothesis that increased mucus accumulation is a function of disease status (RAO) and the environment. Further, that increased mucus accumulation is associated with neutrophilic airway inflammation and the age of the horse (chapters 2-4).

- used the microrheometric method to test the hypothesis that increased mucus accumulation is a result of and associated with unfavorable alterations of mucus physical properties, i.e., increased viscoelasticity and decreased predicted clearability (chapters 5-6).

- identified equine homologues of gel-forming mucin genes and measured mRNA levels in conjunction with morphometric methods to test the hypothesis that increased mucus accumulation is a result of and associated with increased production of mucus (chapters 7-9).

The individual chapters are written and referenced so that they can be read independently. At the beginning of chapters 2-8 describing the original research, I briefly state the thoughts behind the individual study in the context of my thesis. The studies themselves are then reported in the first-person plural form, i.e., "we performed this study," since indeed I did not perform these studies alone, but with the help of many others, and also because I had already written up some of the work as research papers.

CHAPTER 1

LITERATURE REVIEW

Nature of airway mucous secretions

Airway mucous secretions are a heterogeneous mix of water, electrolytes, lipids and proteins, as well as cells and cellular debris [4]. All these components form the complex biofilm most often called "mucus". Of particular functional importance are mucins, however. More strictly defined, respiratory mucus represents only the gel-forming mucin products derived from secretion of epithelial goblet cells and submucosal glands. Tracheobronchial secretions, on the other hand, include the mucus plus all the other components listed above. For the purpose of this work, the terms "mucus (accumulation)" and "respiratory (or airway) secretions" are used interchangeably to describe the entity of gel-like secretions with all their components.

Respiratory mucus has important physiological roles: trapping, inhibition, destruction and removal by mucociliary clearance of inhaled materials as well as humidification of air and prevention of fluid loss. Excess mucus accumulations may enhance some of these physiological functions. They can also lead to undesired functional consequences, such as airflow obstruction, cough and bacterial colonization. As in human chronic bronchitis, asthma and cystic fibrosis and in animal models of these diseases, mucus accumulation in horses is generally viewed as a characteristic, but non-specific sign of various airway diseases [5]. In this review of the literature and in the following investigations, I will focus on equine RAO and IAD.

Description, definitions and significance of RAO and IAD

Description and definitions–Non-infectious lower airway disease is a major health problem of stabled horses. In conventional stable environments in which horses are bedded on straw and fed hay, the air in the breathing zone of horses contains very high levels of organic dust with allergens and non-specific irritants like endotoxin [6-8]. Horses housed permanently indoors in such a conventional stable environment show high incidences of non-infectious lower airway disease. Published estimates range from 54% in Switzerland [9] to 79% in the Netherlands [10]. Thus, environmentally induced equine lower airway disease is a major health problem of horses, and understanding the underlying mechanisms is crucial for the improvement of environmental and medical management. Because the use of various terms referring to environmentally-induced equine lower airway disease has led to confusion, the state-of-the-art of our knowledge was reviewed and working definitions were proposed in two recent workshops [11, 12]. RAO refers to the severe, debilitating disease characterized by clinically evident increased breathing effort due to cholinergic bronchospasm, coughing, and airway hyperreactivity as well as neutrophil and mucus accumulation in the airways [1, 3, 13-22]. In most RAO-affected horses clinical exacerbation is induced by stabling and hay feeding, and clinical remission is often achieved within days to weeks when horses are kept at pasture or given corticosteroids [1, 23-28]. RAO has been well characterized, and pathogenic mechanisms involved in this disease show striking similarities to human asthma.

In contrast to horses with RAO, however, many horses show less severe forms of lower airway disease without clinically evident increased breathing effort when exposed to a conventional stable environment. These horses are now categorized as having IAD

[29], which may be associated with airway inflammation, mucus accumulation and airway hyperreactivity and clinically manifest with cough and exercise intolerance [18, 29, 30]. The pathogenesis of IAD is much less well understood than that of RAO, but the above-cited surveys [9, 10] suggest that it is very common among stabled mature horses.

Clinical significance–Signs of RAO in exacerbation are obvious and its clinical significance as a debilitating disease is undisputed. IAD, on the other hand, may often go undetected unless one specifically looks for its signs. The clinical significance of IAD as a cause of "poor performance" is far from clear. In a standardized exercise test to fatigue in Thoroughbred and Standardbred racehorses with poor performance, the predicted exercise capacity correlated best with indices of oxygen uptake and transport capacity [31]. Even mild airway disease leading to peripheral airway obstruction and ventilation/perfusion mismatching that is subclinical at rest, may significantly compromise oxygen uptake during maximal exercise. However, the percentages of "poor performance" cases attributable to IAD in clinical studies differ widely. Several authors [32-35] cite high incidences of 22-50% of IAD in racehorses. In contrast, Morris and Seeherman (1991 [36]) diagnosed only 4% of 275 "poor performance" cases as lower airway disease based on thoracic radiographs and ventilation / perfusion scintigraphy. In another study of 348 cases of poor performance, IAD (or lower airway disease) was not even mentioned, while upper airway, cardiac and musculoskeletal problems constituted most of the 73% of cases in which a definitive diagnosis was reached [37]. The same group, however, found a high proportion of IAD and/or EIPH in 42 "poor performance" horses in another study [35]. Rather than indicating the true causative effect of IAD, these wide variations seem to reflect the complex nature of "poor performance",

Table 1-1: Characteristics of recurrent airway obstruction (RAO) and inflammatory airway disease (IAD)

	RAO	IAD
Typical signalment	Older (> 8 years of age) horses; no gender or known breed preference, but a genetic predilection has been demonstrated	In particular, younger racehorses, but also horses of any age, use and breed.
Causes	Allergens (e.g. mold spores) in combination with non-specific irritants from hay dust and indoors stable environment	Indoors stable environment; Possibly bacterial colonization especially in younger horses; otherwise largely unknown
Main clinical signs	Overt respiratory distress and coughing when stabled and exposed to hay	Coughing, poor performance; may not show any clinical signs
Main additional diagnostic characteristics	Bronchospasm is reversible with bronchodilator; >20% neutrophils in BALF; increased endoscopic mucus grades	>5% neutrophils in BALF; increased endoscopic mucus grades; increased responsiveness to inhaled histamine or metacholine
Main pathophysiological characteristics	Cholinergic bronchospasm; neutrophilic inflammation; moderate to large mucus accumulation in trachea	Neutrophilic, eosinophilic or mast cell -type inflammation; moderate to large mucus accumulation in trachea; airway hyperresponsiveness
Treatment	Avoid dust exposure; Corticosteroids; bronchodilators	Avoid dust exposure; various treatments of unproven efficacy

Broncho-alveolar lavage fluid (BALF)

diagnostic difficulties, and the specific focus of the respective study. The role of IAD and associated excess airway mucus in causing "poor performance" is thus presently unclear.

Despite the confusion on definitions and the conflicting data on significance, it is clear that mucus accumulation is generally regarded as a hallmark of both RAO [3, 11] and IAD [12, 32, 33] and is often attributed a causative role in airway obstruction.

Clinical and pathophysiological significance of mucus accumulations: Is the phenomenon worth studying?

Increased mucus accumulation can directly cause bronchial obstruction as well as effectively increase resting airway wall thickness. This latter effect amplifies to the 4^{th} power the lumen-narrowing effect of bronchoconstriction [38]. That is, the same amount of mucus will for simple geometrical reasons obstruct the lumen increasingly as the inner diameter of the airway is decreased by contraction of the surrounding airway smooth muscle. The logic of this argument is simple and convincing, and the obstructive effect of excessive secretions seems intuitively clear. The available data, however, presents a much more complex and confusing picture.

Comparative aspects–The volumes of excessive airway mucus accumulations, i.e., sputum, and its unfavorable rheological properties are thought to be associated with the severity of human airway diseases. Mucus accumulation may contribute to the morbidity of human airway diseases by causing patient discomfort, airflow obstruction, and predisposition to respiratory infection [39]. In large population based studies, the presence of chronic mucus secretion is associated with excess decline in forced expiratory volume in one second [40] and with an increased risk of mortality [41]. However, the magnitude of a direct causative effect of excessive airway mucus on airway

obstruction, patient morbidity and mortality, as well as the benefit of "mucoactive therapy" are all controversial and, at best, difficult to assess [39]. Less controversial than the significance of excess secretions for airway obstruction, is their role as a predisposing factor for pulmonary bacterial infections in humans. Exacerbations in patients with cystic fibrosis and chronic obstructive pulmonary disease are associated with colonization of mucus by *H. influenzae, M. catarrhalis, S. pneumoniae, H. parainfluenza*, and, less commonly, *P. aeruginosa* and *K. pneumoniae* [39].

Significance of mucus accumulation in equine lower airway diseases– Following administration of bronchodilators to RAO-affected horses in clinical exacerbation, airway resistance rapidly decreases, but still remains greater than in control horses [42, 43]. In another study, an inhaled bronchodilator improved the distribution of radionucleotide aerosol in all tested horses with acute RAO, but in 5 of 6 affected horses some degree of airway obstruction was still detectable [44]. Robinson *et al.* (1996 [3]) suggested that these remaining lung function deficits and the inconsistent effect of bronchodilators on dynamic compliance may be due to mucus accumulation and airway wall thickening. For lack of a reliable and practical way to quantify mucus accumulation its relative contribution has not been defined, however.

In one study the volumes of secretions aspirated transtracheally (obviously without prior infusion of saline) were directly measured. Four horses without lower airway disease had less than 1ml aspirable mucus. Out of 22 horses with IAD or RAO 5 had < 1ml, 3 had 1-3 ml and 15 had > 3ml of tracheal secretions [45]. Since then, only subjective endoscopic scoring of mucus accumulation has been employed to quantify mucus accumulation. Various systems with scores from 0-3 [18] and 0-5 [46, 47] have been used.

Endoscopic mucus scoring has yielded interesting results. In a retrospective clinical study, Dixon et al. (1995 [5]) clearly demonstrates the non-specific nature of mucus accumulation in horse airway diseases: compared with a group of healthy control horses, mucus was increased in RAO (n=148), "undifferentiated pulmonary disease" (n=18), "infectious pulmonary disease" (n=45), "S. zooepidemicus pulmonary infection" (n=7), "Lungworm infestation" (n=7), "exercise-induced pulmonary hemorrhage" (n=16) and "miscellaneous pulmonary disease" (n=20). RAO horses showed the highest score (median score of 3), but this was not significantly different from all the other groups. Mucus scores were also increased in RAO-affected horses compared with IAD and with clinically healthy animals [46, 48]. Furthermore, endoscopically observed increased mucus accumulation is associated with coughing [49-51], decreased racing performance [33] and decreased lung function [48].

Increased mucus accumulations are also associated with bacterial colonization of the airways. When horses are prevented from lowering their head for a prolonged period of time, mucus pooling with excessive bacterial growth occurs in the distal trachea [52]. This may increase the risk of bacterial pleuro-pneumonia in immunocompromized animals. Furthermore, inflammation scores, which include mucus accumulation, are highly correlated with positive bacterial cultures from tracheobronchial secretions in IAD of young racehorses [53]. Molecular interactions between bacteria and mucin glycoproteins are a highly interesting field, which is only beginning to be explored [54]. However, although even in healthy horses *S. zooepidemicus* and other upper airway commensals are frequently isolated from tracheal secretions [55], exacerbations associated with bacterial infections are not a clinical problem recognized in RAO-affected horses [11].

Based on the available information, I conclude that mucus accumulation is a clinical problem in IAD, RAO and other lung diseases, can directly and indirectly affect lung function and performance and can be associated with bacterial colonization. I also note, however, that the magnitude of these effects is unclear, that the effect on performance has not been studied in sport horses, and that endoscopic scoring has not been validated. No one grading scale is generally accepted, and, therefore, comparisons, between studies and observers are difficult at best. In some studies a mucus score is compounded into a general examination score based on various other parameters [53], and it is therefore impossible to extract mucus specific information. The repeatability of mucus scores both within an individual horse and within and particularly between observers may be problematic in a measure that is both variable and subjective by nature, for example, a horse may cough and clear its trachea of mucus, exercise could increase or reduce visible mucus, and "a few" versus "many" mucous flakes may not mean the same to different observers.

The diagnostic potential of a standardized mucus score and, possibly immunological tools for quantifying mucins [54], must be investigated and validated. The significance of mucus accumulation in sport horses should be evaluated. Moreover, in order to better understand, and ultimately treat this phenomenon, its causes and underlying mechanisms must be addressed.

Causes and mechanisms of airway mucus accumulation

The amount of mucus present in the lumen of airways is the result of a dynamic process that involves specific mucin gene expression, the production, storage and secretion of mucins into the airway lumen, and the subsequent clearance by cilia lining

the conducting airways. In addition to mucins, all other components of mucous secretions can be present in excess, and must also be removed by mucociliary clearance. Clearance capacity, probably genetically determined, can therefore be reduced by alterations in the ciliary apparatus and/or in mucus physical properties that influence its clearability.

This is a simple concept, but our understanding of its fundamental cellular and molecular regulatory mechanisms in normal and diseased airways is as yet very incomplete. Although production logically precedes clearance, I will discuss the latter first, since more is known about it in the horse.

Clearance: primarily compromised or just overwhelmed?–Mucociliary clearance in humans increases from the periphery to the central airways, is reduced during sleep and decreases with age. Physiologic regulation is primarily exercised by autonomic mechanisms: β-adrenergic agonists are potent stimulants of clearance, and cholinergic antagonists potent depressants, while the effect of cholinergic agonist drugs is variable [56].

In healthy horses, physiologic regulation of clearance appears to be similar to other species. While atropine (0.02 mg/kg IV) significantly decreases tracheal clearance rate [57], a β_2-agonist (Clenbuterol 0.8 μg/kg IV) significantly increases tracheal mucus velocity in vivo [58]. Water vapor-saturated air therapy, exercise and mild dehydration by furosemide have no effect on mucociliary clearance in healthy horses [59]. Gravitational force can decrease or accelerate tracheal mucus velocity in vivo [60] and in vitro [61].

A variety of airborne pollutants, such as SO_2, NO_2, O_3 and smoke from cigarettes, have deleterious effects on mucociliary clearance. After cessation of smoking, mucociliary clearance improves after 3 months [56]. In donkeys, exposure to sulfuric acid

(H_2SO_4) leads to a decreased mucociliary clearance that takes up to three months to return to normal [62].

Humans with chronic bronchitis, emphysema, CF and asthma can have reduced rates of clearance. A single allergen challenge reduces mucociliary clearance rate by 28% in asthmatics, and by 46-64% for 7 days in sheep [56, 63]. Airway inflammation appears to play a major role in this decline of mucociliary clearance [63]. Similarly, a decrease in mucus clearance associated with airway inflammation could contribute to the accumulation of airway sections in horses with allergic lower airway disease [2].

Investigations of the effectiveness of mucociliary clearance in RAO-affected horses have yielded conflicting results. Clearance has been determined by use of a radioactive marker that was followed up the airway by a scintigraphic method. In RAO-affected animals, clearance was decreased by 24% compared with controls, and radioactive marker was less likely to move as a discrete bolus [64]. Another investigation that tracked the movement of a surface marker by use of endoscopy found a tracheal mucus velocity of 11.41±1.09 mm/min in RAO-affected animals vs. 21.41±1.03 mm/min in controls [58]. Others, in contrast, found no difference between RAO-affected and healthy horses [65]. No studies have investigated mucociliary clearance rate in IAD. Infection with rhinovirus has no effect on mucociliary clearance, but herpesvirus reduces clearance in half of the infected horses. In contrast, mucociliary clearance is severely depressed in all horses after influenza infection and takes four weeks to return to normal [66], underlining the importance of adequate rest periods and good air hygiene after clinically apparent respiratory viral infections.

The studies described in the previous paragraph also found a wide variation of transport rates among healthy control animals. Validation of an endoscopic marker

method showed good repeatability within 20 healthy horses, but considerable differences between individual horses [67]. Such wide interindividual variation also has been observed in humans, and twin studies suggest an individual, genetically determined mucociliary clearance capacity [68].

When the amount of mucus exceeds the mucociliary clearance capacity, secretions progressively accumulate, and must then be cleared by coughing [69]. The association of excess mucus accumulation and cough has been shown in a clinical study in horses [49]. In airway disease, mucociliary clearance capacity may be compromised due to changes in the ciliary apparatus or unfavorable physical properties of the mucus gel [2, 69].

Ciliary function and dysfunction–Ciliary amplitude and beat frequency determine the maximal velocity at the tips of the cilia, and hence the maximal forward force transmitted to the mucus layer. The density of spacing between the cilia will also affect clearance rate, since more energy is lost with increasing distance between the cilia. In smaller airways, the cilia are generally shorter, and less densely packed than in the large bronchi, which partly explains the decrease of transport velocity from the central airways towards the periphery.

Ciliary dysfunction in disease can result in severe mucus stasis and loss of the natural defense against inhaled particles. Immotile cilia and ciliary dyskinesia syndromes in humans demonstrate the potentially lethal effects of ciliary dysfunction [56]. These congenital diseases have not been diagnosed in the horse. Respiratory viral diseases, however, can also seriously affect ciliary function. In humans, reduced mucociliary transport rate following viral infections correlates with the presence of acquired ciliary defects. About 50% of cilia must be lost or damaged to cause a measurable decrease of

clearance [56], indicating the high reserve capacity of the ciliary apparatus. Differences in severity of epithelial ciliary damage caused by rhino-, herpes- and influenza viruses, explain the differences in mucociliary clearance decrease observed after infection with these agents [66].

Compared with viral diseases, the extent and significance of damage to the ciliary apparatus is less clear in chronic non-infectious airway diseases of human [56] and horse: Kaup *et al.* (1990 [70]) reported a loss of ciliated cells and abnormal ciliary structure in large conducting airways of RAO-affected horses. Undifferentiated cells replaced ciliated cells in a hyperplastic epithelium. These changes differ greatly in their severity and extent between RAO-affected horses. While the extent of ciliary loss is associated with the severity of clinical disease, the degree of ciliary malformations appears unrelated to disease state and severity. Galati *et al.* (1991 [71]) point out that such structural ciliary defects occur in about 5% (a frequency comparable to other species) of cilia in airway epithelium of healthy horses. Considering the high reserve capacity of the ciliary apparatus, the clinical significance of the changes observed in non-viral lower airway diseases is unclear.

Studies on excised pieces of trachea from healthy horses have shown that variations of ciliary beat frequency at physiological temperatures have only a minor effect on mucociliary clearance rate. In contrast, physical properties of mucus are very important [72].

Mucus biophysical properties and their alterations in disease–Mucous factors affecting mucociliary clearance are the depth of the mucous layer and its biophysical properties. Mucus that is deep, i.e., more than the physiological 5-10 microns, is well suited for cough clearance, but unfavorable for mucociliary transport [69]. Mucus

biophysical properties can be characterized and quantified as visco-elasticity, spinnability and adhesivity. All of these affect clearance, but viscosity and visco-elasticity are the best studied in respiratory pathophysiology. They are also the only mucus physical properties that have been investigated in the horse.

A balance between viscosity and elasticity must be maintained for optimal mucociliary clearance: The elasticity of mucus is important for clearance by cilia because elastic mucus efficiently transmits energy. The viscosity of mucus results in energy loss, but is necessary so that mucus can be displaced. The essential visco-elastic nature of mucus is a result of its three-dimensional, cross-linked mucin network structure. Fig. 1 illustrates how this three-dimensional network is in turn dependent on a number of molecular forms of bonding: 1) disulfide bonds join glycoprotein subunits into extended macromolecular chains; 2) because of their size, mucin polymers readily form entanglements among each other; 3) oligosaccharide sidechains, which make up 80 % of the mucin weight, form hydrogen bonds with complementary sugar units on neighboring mucins; 4) ionic interactions (negatively charged sugar units and positively charged amino acids) extend the macromolecular conformation; 5) inflammation can add an extra network of high molecular weight DNA and actin filaments released from dying leukocytes. Interactions with water, electrolytes, hydrogen ions (pH), lipids, enzymes and other (serum) proteins will influence these mucin gel-bonds, resulting in the physical properties of mucus.

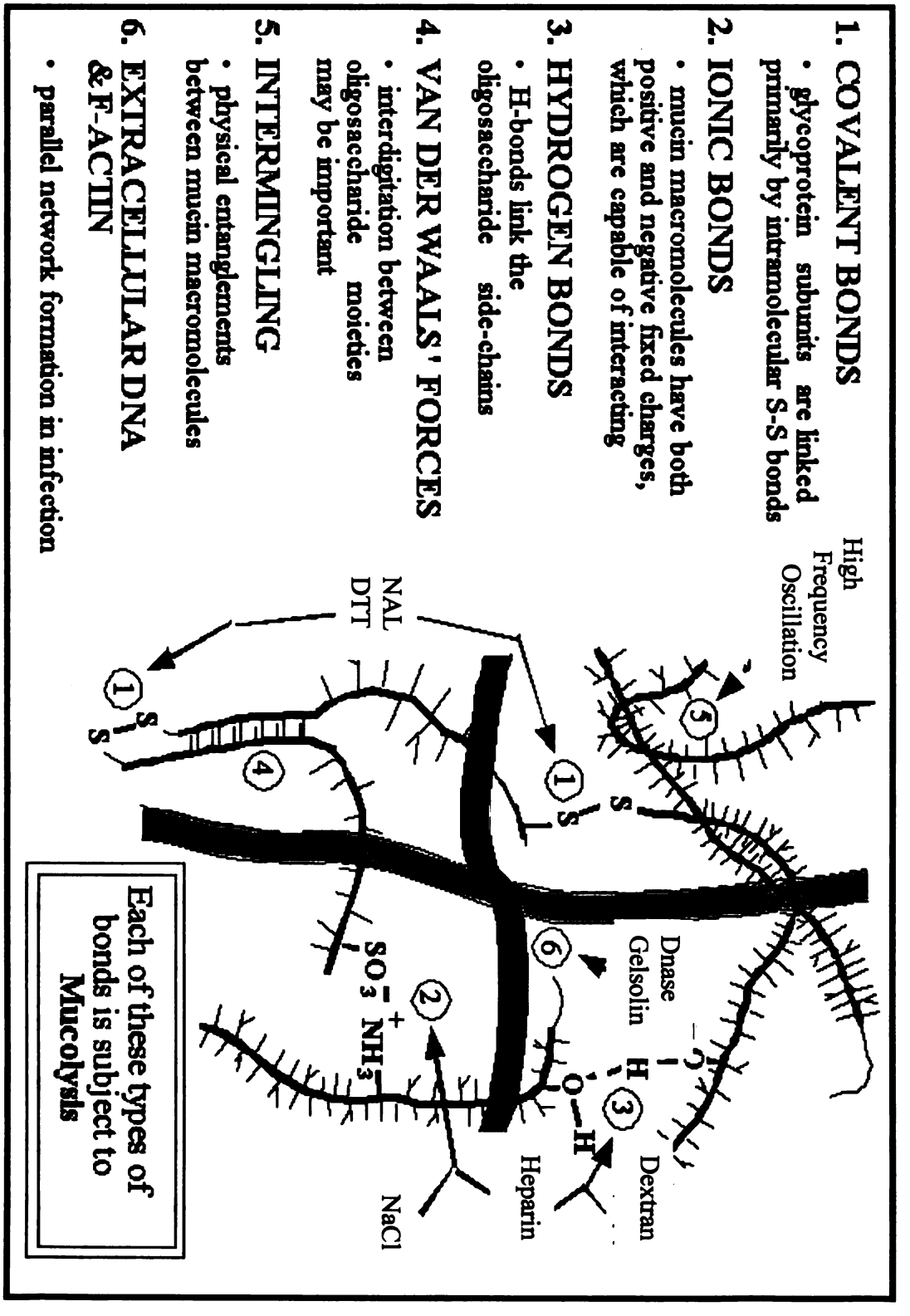
The sheer complexity of this system makes it a difficult object of study. Furthermore, healthy airways contain only small amounts of secretions available for sampling and analysis. Only the development of methods that allow for the investigation of microliter quantities of mucus–microrheometry (King and Macklem, 1977)–has made

the comparison of "diseased" with "healthy" mucus possible [73]. Hence, the literature is still heavily biased by data from disease states without healthy controls. This helps explain why the role of unfavorable rheological changes in chronic bronchitis, cystic fibrosis and asthma is acknowledged, but its importance is still controversial. Patients suffering from these diseases may have mucus of very high, normal or, uncommonly, low viscoelasticity. Overall, high viscoelasticity is associated with increased purulence of secretions [39, 69].

Not surprisingly, the available data on mucus rheology in equine airway disease is far from complete or conclusive. Based only on the physical appearance of tracheobronchial secretions, Schatzmann *et al.* (1972 [45]) proposed that mucus viscosity increases with the severity of clinical signs of airway obstruction in RAO-horses. However, when these authors measured viscosity of mucus in 5 RAO-affected horses they found it to be lower than in human patients with severe chronic bronchitis [21]. Hajer (1979 PhD thesis, cited in [58]) found increased viscosity of tracheal mucus in RAO horses. No study has directly compared mucus from RAO-affected to that of healthy horses, however.

It is important to note that there is indirect evidence that factors other than viscoelasticity may negatively influence clearability in RAO-affected horses. Adhesivity and wettability, which contribute to the optimal interface properties of mucus, are primarily influenced by its phospholipid content and composition [74]. Exogenous surfactant increases transport velocity ex vivo [75] and in vivo [76]. Surfactant is present in amounts sufficient to significantly reduce surface tension in the trachea of healthy horses [77], but clinical observation (reduced foam of BAL) and biochemical analysis of airway secretions suggest that a deficit and altered composition of surfactant are present in RAO [78], and after horses have been subjected to long periods of transport [79].

Fig. 1-1: Types of bonds in a mucous gel and respective mucolytic agents
Entire figure from M. King, Pulmonary Research Group, University of Alberta, Canada.



Our current understanding of equine mucus rheology in the horse is patchy at best, but it provides a rational basis to hypothesize that unfavorable mucus rheology is a cause of the decreased mucus clearance in RAO-affected horses. Characterization of mucus rheology in RAO and investigation of underlying pathophysiological mechanisms should ultimately lead to better treatment. I therefore set out to compare viscoelasticity between RAO-affected and healthy control horses before and during environmental challenge.

Production and secretion–The physiologically heterogeneous mucus is a mix of water (~95%), electrolytes (1%), lipids (1%), proteins (2-3%): enzymes, other (serum) proteins and high molecular weight O-linked glycoproteins referred to as mucins, as well as very variable amounts of epithelial and inflammatory cells [4]. There is very little published information on the relative proportions of these components in health and disease [80]. Nonetheless it is clear, that the concentration of all of these components can be altered, and all these alterations will affect the biophysical properties and, consequently the rheology of mucus. In contrast, only an increase in inflammatory cell influx, serum protein exudation and mucin secretion can be expected to significantly increase the volume of the secretions to result in their accumulation. Proteins, in particular mucin glycoproteins, will influence the total volume by raising oncotic pressure, i.e., drawing and retaining water in the mucus gel. Inflammatory cells may directly contribute to the volume of mucus, i.e., "mucopus".

The BALF total cell counts are consistently increased during RAO exacerbations. The quantification of cells or substances in the pulmonary lining fluid of the lower respiratory tract, as obtained by BAL, is not precise because of the variable dilution of

the ELF by the instilled lavage fluid. In order to estimate the number of cells per volume of ELF, cell numbers have been adjusted to the concentration of albumen or urea. In horses, the total cell count per volume of ELF is moderately higher in RAO-affected animals (approximate median 45'000 cells / µl) than in controls (~30'000 cells / µl), when adjusted for albumen, but slightly lower (~17'000 cells / µl in RAO vs. ~20'000 cells / µl in controls) when adjusted for urea concentration [81]. These results do not indicate substantial differences in cell content of airway secretions. The use of urea and albumin concentrations in such calculations is fraught with methodological problems, however [82]. Furthermore, direct microscopic inspection of undiluted RAO tracheo-bronchial secretions often shows a high cellularity compared to mucus sampled from control horses, suggesting that the volumetric contribution of inflammatory cells to mucopurulent (*sic!*) airway secretions may be substantial.

Persson (1995 [83]) proposed that airway inflammation causes plasma exudation from postcapillary venules, earlier than and independent from cellular extravasation. According to this model, the exudate causes a bulk flow of non-sieved plasma into the airway lumen rather than airway wall edema. After activation of proteolytic cascades, the multiplication of plasma-derived protein molecules attracts and retains progressively more fluid, thereby further increasing the volume of the mucus. Winder and von Fellenberg (1986 [84]) immunohistochemically demonstrated extravasated fibrinogen in tissues of horses with RAO, indicating that plasma exudation does take place in RAO. Lower BALF albumen: urea ratios in RAO than in controls [81], however, speak against significant plasma exudation, which should increase albumen relative to urea.

While the relative contributions of cellular and plasma protein components to the volume of airway mucus secretions in RAO are presently unknown, I will focus on the main constituents of mucus, the large, cross-linked mucin glycoproteins.

Mucins: production and secretion–Mucins, also called mucous glycoproteins, are high molecular weight glycoconjugates. Numerous oligosaccharide side chains are O-glycosidically linked to threonine and serine of the peptide core, which represents the primary gene product, the apomucin. The threonine- and serine-rich regions are formed of tandem repeat sequences, which are the common feature of epithelial mucins. By convention, human mucin genes (products) are designated by MUC, mouse by Muc, and rat by rMuc. Epithelial mucins are subdivided into membrane-associated and secretory products. MUC7, a salivary mucin, is the only soluble secretory form, while all other secretory mucins identified so far contain non-repetitive cysteine-rich regions, believed to allow intra- and intermolecular cross linking through disulfide bond formation. The major gel-forming mucins expressed in human and rodent airways (MUC2, MUC5AC and MUC5B) are encoded by members of a large family of mucin genes [85-87]. Also present are highly glycosylated, monomeric MUC1 and MUC4 mucins shed from the cell surface as well as additional N and/or O-glycosylated proteins, lipids, and glycolipids [88]. Synthesis, storage and release of mucins are central properties of the innate mucosal defense against inhaled irritants and pathogens.

"Too much of a good thing" aptly describes the situation where the airway mucosa produces and releases so much mucin glycoprotein as to overwhelm the capacity of the mucociliary transport system. Such a hypersecretory state is found in human chronic bronchitis, asthma exacerbation and cystic fibrosis [89]. MUC5AC and MUC5B are the major oligomeric mucins and airways mucus contains variable amounts of these

glycoproteins [90-92]. By contrast, the MUC2 mucin comprised only 2.5% of the weight of the gel-forming mucins, indicating that MUC2 is a minor component in human sputum. Finally, the amounts and glycosylated variants of the MUC5AC and MUC5B mucins can be altered significantly in diseased airways with, for instance, an increase in the low-charge form of the MUC5B mucin in cystic fibrosis and chronic obstructive pulmonary disease mucus [90-92].

MUC5B is expressed predominantly in submucosal glands, and an increase is thought to occur through gland enlargement [93]. One study found up-regulation of MUC5B expression in a mouse model of asthma [94], but others report that this mucin is constitutively expressed and does not directly respond to irritant and inflammatory stimuli [95]. In contrast, many studies have shown MUC5AC up-regulation in response to various stimuli and mediators -notably BAL from asthmatics- has been shown in a variety of models of airway inflammation [95, 96]. MUC5AC expression also precedes and accompanies epithelial mucous cell metaplasia [97]. Increased expression of MUC2, normally a predominantly intestinal mucin, may play a role in cystic fibrosis [98, 99]. Presently, MUC5AC is regarded as the main "signature" mucin in experimental and natural models of airway disease.

Based on reactivity with polyclonal antibodies raised against the respective human mucins, it has been proposed that a homologue of MUC5AC is present in equine stomach [100], and that homologues of MUC5AC and MUC5B may be found in equine airway secretions [101, 102]. These findings are based on reactivity with antibodies that have been raised against human mucins, however, and genetic identification of equine homologues of the main gel-forming mucins is a priority for the investigation of mucin hypersecretion in RAO.

Jefcoat et al. (2001 [54]) assessed relative differences in amount of mucin glycoproteins between control and RAO-affected horses using a carbohydrate side chain-specific monoclonal antibody (4E4) in an enzyme-linked immunosorbent assay, and by carbohydrate-specific enzyme-linked lectin assays. Significantly increased levels of 4E4-immunoreactive glycoprotein and the mucin-associated carbohydrates fucose (α-1,2 linkage), N-acetyl-glucosamine, and N-acetyl-galactosamine were detected in RAO horses in acute disease. RAO horses in remission also showed increased levels of 4E4-recognized mucin, α-1,2 fucose, and N-acetyl-glucosamine, while N-acetyl-galactosamine levels were equivalent to those of controls. Although not measures of absolute amounts of mucin glycoproteins or mucin carbohydrates in the airways, these ELISA and ELLA assays show persistent alteration of mucin oligosaccharide side-chains in RAO-affected horses. Mucin oligosaccharide side-chain structure has functional importance, imparting specific binding activity toward structures such as bacterial adhesin molecules [103, 104], and contributing to the degree of viscoelasticity of the mucus layer [69, 105]. Individual mucin sugars, such as fucose, have also been used as markers of tracheobronchial mucus production in humans [106], and α-1,2 fucose may be associated with MUC5AC, in particular (Jefcoat, personal communication).

Origin and mechanisms of mucin hypersecretion–Mucin gene products are produced and secreted by epithelial mucous secretory goblet cells, which produce MUC5AC and MUC5B, and by submucosal glands, which produce mainly MUC5B [107]. In the horse, submucosal glands are sparse even in the large airways [108], while the submucosal glands can contribute a large fraction of total secreted mucins in humans. In natural and experimental airway diseases, increased amounts of luminal airway mucus are correlated with the histological appearance of numerous goblet cells in the surface

epithelium and submucosal glands in airways that normally contain only some mucous cells (i.e., mucous cell hyperplasia) and in regions that normally contain few or no mucous cells (i.e., mucous cell metaplasia) [97, 109-111].

Several histopathological and ultrastructural studies describe mucous cell hyperplasia, metaplasia, and hypertrophy in pulmonary airways of RAO-affected horses (e.g., [70]; reviewed in [2]). None of these reports are based on quantitative morphometric techniques, however. In contrast, Hotchkiss (1998 [112]) reported preliminary morphometric results that surprisingly showed no significant differences in the amount of stored mucosubstance between RAO and control horses at any airway generation.

Such measurements are based on the assessment of "a moment in time" within the dynamic process of mucin production, storage, and secretion, however. Current understanding of inflammatory airway pathogenesis indicates that after injury, immediate release of stored mucosubstance is followed by up-regulation of mucin production within hours, and an increase in goblet cell numbers within days [113].

Since horses have few submucosal glands and MUC5AC appears to be the main inducible mucin of goblet cells, I propose that in RAO-affected horses the bulk of secreted mucins are MUC5AC products derived from surface epithelial goblet cells [2, 70]. Excessive amounts of airway mucus in RAO-affected horses could thus be due to elevated production and secretion of mucins by the same number of mucous cells found in normal horses, i.e., increased production and secretion per cell. Alternatively, there may be increased numbers of airway epithelial mucous cells without a change in production or secretion per cell. Concurrently, total volumes of stored mucosubstance in the epithelium can be increased or decreased. Storage may increase especially during

clinical remission and "low-level secretion", while it may, at least temporarily decrease in "high-through-put states" during acute exacerbation.

Control of mucus (hyper)secretion can be further divided into many more steps, involving both transcriptional and post-transcriptional mechanisms [96] that regulate mRNA formation and degradation (stability), protein translation and carbohydrate modification at the molecular level; granule formation and exocytosis on the cellular level, as well as the above discussed changes on the epithelial level. Furthermore, these mechanisms must be viewed in the pathophysiological and, in particular, the immunological context of the disease.

Pathophysiological and immunological context of mucus hypersecretion–*Innate immunity* provides broad, but relatively nonspecific host defenses [114]. Macrophages and neutrophils as well as epithelial cells are regulator and effector cells of the innate inflammatory response. Two of the most important early-response cytokines in innate immunity are interleukin (IL)-1β and tumor necrosis factor (TNF)-α. While toll-like-receptors (TLRs) mediate pathogen recognition by the innate immune system, cytokines are necessary for the full development of the innate host defense and the transition to adaptive immunity.

The "yin-yang" T helper(h)2–Th1 hypothesis postulates two distinct patterns of cytokine secretion by stimulated CD4+ T cells [115]. The lists of known Th1 (interferon [IFN]-γ IL-2, -12, -18 and TNF-α) and Th2 (IL-4, -5, -6, -10, and -13) cytokines continues to expand, and some of these cytokines are not exclusively produced by one type of Th cells, but also by other T cells and cells unrelated to leukocytes. Th1 type cells primarily confer immunity against intracellular pathogens, whereas Th2 cells mediate responses against extracellular parasites [116]. Th2 type cytokines are also key factors in

asthma and experimental models of allergic airway disease [117-119]. Like innate immunity reactions, Th1 responses can be initiated by inhalation of microbial pathogens, endotoxin and particulate material in organic dusts. Th1 type profiles are observed in human chronic bronchitis. Both Th1 and innate immunity may oppose Th2-mediated responses, but can also exacerbate them or cause inflammatory airway disease in their own right [120].

Specific profiles and polarization (Th1 vs. Th2) depend on the composition, timing, dose and route of the challenge as well as the genetic background of the species, breeds and strains investigated [121-124]. For instance, in (early) asthma Th1- and Th2 type cytokines are often co-expressed [125] and their profiles as well as the associated pathophysiological and morphological features overlap in people suffering from both chronic bronchitis and asthma [126].

Most cytokines play pleiotropic roles in down-stream effector pathways with functional divergence, convergence, "cross talk" and redundancy [127-129]. IL-13, in contrast, has been identified as a critical regulator and very direct effector of the allergic response (hyperresponsiveness; mucus secretion) in animal models [127, 130]. Recently, a calcium-dependent chloride channel (CACC1; gob-5 in the mouse) has been identified as an important down-stream effector element of IL-13, mediating airway hyperresponsiveness and overproduction of mucus in animal models and, possibly, in asthmatic subjects [131-133]. IL-13 as well as Th1-type and innate immunity cytokines can also up-regulate and activate epidermal growth factor receptor (EGFR) through epidermal growth factor (EGF), transforming growth factor (TGF)-α and other ligands. Neutrophils can up-regulate EGFR expression through TNF-α and subsequently activate EGFR in a ligand-independent fashion through the release of oxidative radicals [134,

135]. TNF-α can also directly increase MUC5AC mRNA half-life, indicating that transcript stabilization in addition to up-regulation of expression may be an important mechanism leading to increased MUC5AC mRNA levels. In the bronchial epithelium, both CACC1 and EGFR pathways converge on up-regulation of MUC5AC, the gel-forming, dominant mucin gene expressed in goblet cells, with subsequent mucous remodeling and hypersecretion [136]. Endotoxin and other microbial components can potentiate this effect through toll-like receptors (TLR-2 and-4) [137] and activation of the NF-κB pathway [138]. Less well studied, but as important as the induction of mucin hypersecretion, is what happens afterwards: There is evidence that the Th2 cytokine IL-13 delays apoptosis of goblet cells leading to persistence of mucus cell metaplasia, while the Th1 cytokine IFN-γ leads to the resolution of these Th2-associated changes [139, 140].

Much insight regarding mucous secretory cell biology has been gained in rodent models, and the above cited *"yin-yang"* example of regulation is very appealing. Nonetheless, extrapolation to other species demands caution. It is important to recognize that mice and many rat strains normally have few mucous secretory cells in their trachea, bronchi, and bronchioles that are instead dominated by a Clara cell lineage system [141, 142]. Conversely, in human beings [142] as well as in horses [2, 70], mucous secretory cells are found in the surface epithelium of many airway generations. Therefore, more mucous secretory cells in a mouse most likely represents metaplastic conversion of Clara cells into mucous cells, whereas in people and likely in horses, mucous secretory cell hyperplasia is predominant. This important pathogenetic difference may translate to the molecular mechanistic level: For instance, IL-4 and IL-13 induce mucus cell metaplasia and Muc5ac overexpression in genetically manipulated mice [143, 144], but decrease

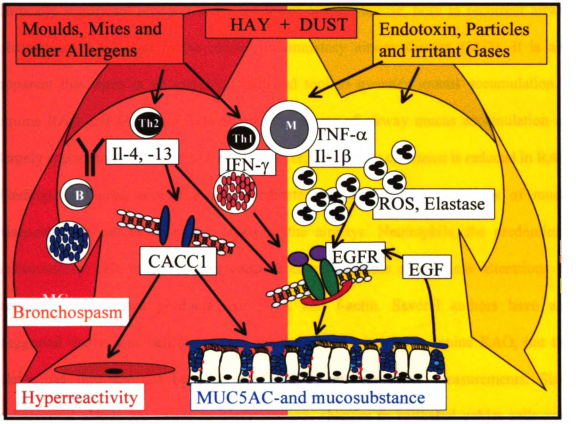
MUC5AC expression in normal human nasal and bronchial epithelial cell cultures [145-147].

Pathophysiological and immunological mechanisms in equine lower airway disease–Cholinergic bronchospasm is directly responsible for most of the measurable (~80%) airway obstruction during RAO crisis [42]. Neural control of mucus secretion is likely less important in the horse than in species with many submucosal glands. Mediators of "neurogenic inflammation" such as substance P, however, may induce mucus hypersecretion in horses [148]. Mast cell mediators increase smooth muscle excitability [149], and airway hyperreactivity is associated with increased mast cell percentages in broncho-alveolar lavage fluid (BALF) [30]. In contrast, neutrophils, the predominant inflammatory cell type in RAO, appear to have no direct effects on bronchospasm [149], but may exacerbate obstruction by inducing mucus accumulation [150, 151]. Neutrophils produce potent secretagogues that are increased in RAO: reactive oxygen species [152], proteases in particular elastase (Jefcoat, personal communication) and LTB_4 [153]. Immunological causes and mechanisms remain ill-defined, but there is evidence for IgE- and IgG- mediated type I and III hyperreactivity [154, 155] against mold allergens, as well as non-specific responses to endotoxin [156]. The full clinical severity and pathophysiological changes can only be reproduced by natural challenge with hay and straw [157]. These findings are in accordance with recent evidence for mixed Th2 and Th1 cytokine profiles in RAO. A Th2 type bias is apparent particularly during crisis: increased numbers of interleukin IL-4 and IL-5 positive (*in situ* hybridization) cells in BALF and increased mRNA levels of IL-4 and IL-13 in BALF [158-160]. However, Th1 type cytokines IFN-γ, IL-1β, TNF-α mRNA levels in BALF can also be increased in crisis and may persist longer during remission [159, 161].

Furthermore, irritants and toxins, in particular endotoxin that is abundant in the stable environment [7], can both directly and indirectly increase and potentiate airway mucin expression and mucus cell hyper- and metaplasia [97, 162, 163], and may therefore play a role in RAO- and IAD-affected as well as in clinically healthy horses.

When RAO horses are removed from conventional stable environment, bronchospasm decreases and clinical signs wane within days. Residual lung function deficits, neutrophil and mucus accumulation persist, however, and are highly correlated with increased NF-κB activity and ICAM-1 expression of epithelial cells [164]. NF-κB is an important intracellular signaling element that is involved in mucin gene up-regulation in vitro [96]. Sustained NF-κB activation in equine RAO is driven by granulocytes and mediated by IL-1β and TNF-α [165], both of these cytokines increase MUC5AC mRNA levels in vitro [166]. While some of the cytokines have been investigated in the horse, the role of epithelial factors in equine RAO is unknown. I propose that EGFR may be central to mucin gene up-regulation in RAO, and that CACC1 could also play a role.

Figure 1-2: My illustrated view of some immunological and pathophysiological mechanisms that may be involved in equine lower airway disease



Some signaling element graphics from J.A. Hotchkiss, Laboratory for Experimental and Toxicologic Pathology, Department of Pathobiology and Diagnostic Investigation, Michigan State University, USA.

Conclusions of literature review and derived hypotheses

The literature review shows that increased airway mucus accumulation is a clinically significant problem in equine lower airway disease, both in recurrent airway obstruction (RAO) and in the milder inflammatory airway disease (IAD). It is also apparent that there is no critically validated tool to quantify mucus accumulation in equine RAO and IAD, and furthermore, the causes of airway mucus accumulation are largely speculative. There is evidence, however, that mucus clearance is reduced in RAO. Findings in human airway diseases indicate that unfavorable alterations of mucus viscoelasticity can cause mucus stasis in the airways. Neutrophils, the predominant inflammatory cell in RAO, can cause both unfavorable rheological alterations by neutrophil breakdown products like DNA and f-actin. Several authors have also suggested that mucus cell hyper- and metaplasia play a role in equine RAO, but this claim has thus far not been substantiated by morphometric measurements. Since submucosal glands are sparse in horse airways, changes in epithelial goblet cells must account for any increased production and secretion of mucins in equine lower airway disease. Gel-forming mucins (MUC2, MUC5AC, MUC5B) have been identified in several other species, and their expression and secretion is regulated within complex immunological networks. Neutrophil products as well as Th1 and Th2 cytokines that are involved in equine RAO can influence the expression of mucins, particularly of MUC5AC, in airway diseases of other species. Recent evidence from experimental models of airway disease further suggests that MUC5AC up-regulation converges on EGFR and CACC1 pathways in the airway epithelium.

Neither mucus accumulation, nor mucus viscoelasticity, nor mucin production and storage have been critically studied in equine RAO. The purpose of my work was therefore to quantify airway mucus accumulation and investigate its causes in RAO-affected compared with clinically healthy control horses.

To achieve this goal, the following hypotheses were developed:

1) Increased mucus accumulation is a function of disease status (RAO) and age of the horse as well as the environment. Further, increased mucus accumulation is associated with neutrophilic airway inflammation.

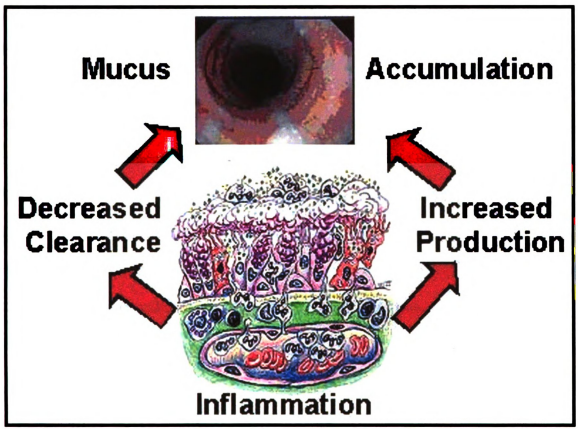
2) Increased mucus accumulation is a result of and associated with unfavorable alterations of mucus physical properties, i.e., increased viscoelasticity and decreased predicted clearability and/or

3) increased production of mucus, i.e., increased mucin gene mRNA levels and storage of mucosubstrance in the airway epithelium.

This dissertation describes: a) the validation of an endoscopic scoring system for mucus accumulation and its use to test the first hypothesis (chapters 2-4), b) the use of the microrheometric method to test the second hypothesis (chapters 5-6), and c) the identification of equine homologues of gel-forming mucin genes and quantification of mRNA levels in conjunction with morphometric methods to test the third hypothesis (chapters 7-9).

The individual chapters are written and referenced so that they can be read independently.

Fig. 1-3: Basic model hypothesis: Mucus accumulation is caused by increased production and/or decreased clearance, in turn these are consequences of airway inflammation



"Angry epithelium" from J.R. Harkema, Laboratory for Experimental and Toxicologic Pathology, Department of Pathobiology and Diagnostic Investigation, Michigan State University, USA

CHAPTER 2

ENDOSCOPIC SCORING OF MUCUS QUANTITY AND QUALITY–OBSERVER AND HORSE RELATED VARIANCE AND RELATIONSHIP WITH MEASURED VARIABLES

Increased mucus accumulation and altered mucus rheological properties have been associated with equine lower airway disease. Endoscopic scoring has been previously used to assess changes in both mucus quantity and quality in horse airways. The validity of such scoring systems, however, has not been investigated.

In order to determine if endoscopic scoring could be used to investigate airway mucus in horses, I performed the following study assessing observer and horse variance of endoscopic scores and their relationship with measured variables.

Abstract

Reasons for performing the study–Endoscopic scoring of airway mucus quantity and quality has not been critically assessed.

Objectives–To evaluate mucus scores for 1) observer- and horse-related variance; 2) association with inflammation, mucus viscoelasticity and measured amounts.

Methods–Variance of scoring within and between observers and over time within horses for mucus accumulation, apparent viscosity, localization and color, and correlations of mucus accumulation scores with neutrophils in secretions were determined. The relationship of accumulation score to measured volumes of "artificial mucus" was investigated. Correlations of mucus accumulation, apparent viscosity and color scores with measured viscoelasticity were tested. Viscoelasticity was compared between ventrally and dorsally collected samples.

Results–Mucus accumulation scoring showed excellent interobserver agreement and moderate horse-related variance, was related to measured volumes of "artificial mucus", and correlated well with neutrophilic airway inflammation. Scores of mucus viscosity, color and localization showed high observer-related variance. Mucus accumulation, apparent viscosity and color scores did not correlate with measured mucus viscoelasticity, but dorsally localized mucus showed 2-fold higher measured viscoelasticity than ventrally localized samples.

Conclusions and relevance–Endoscopic scoring of mucus accumulation is a reliable clinical and research tool. In contrast, apparent viscosity, localization and color scores should be interpreted with caution.

Introduction

Endoscopy is an important tool for both clinical work-up and scientific investigation of equine lower airway disease. Endoscopic scoring systems have been used for subjective, semi-quantitative assessment of accumulation [5, 46] and appearance [46, 167] of airway secretions.

However, both the observer and the subject may be important sources of variance in such scoring systems. For example, "a few" vs. "many" mucous flakes may not mean the same to different observers or a horse may cough before an endoscopic exam and thereby momentarily clear mucus from its trachea. Moreover, relationships between subjective endoscopic scoring variables (e.g., accumulation and apparent viscosity scores) and corresponding measured variables (e.g., mucus volume and viscoelasticity) are unknown. The lack of validation of endoscopic mucus scores makes interpretation of results, comparison between studies and communication between clinicians difficult and uncertain.

The goal of the present study was to evaluate the validity of endoscopic scores used to assess mucus accumulation and character. Specifically, we asked the following questions:

1. Does intra- and interobserver variance limit the usefulness of endoscopic scores?
2. How much do endoscopic scores vary within a horse over the course of a day?
3. Does mucus accumulation score correlate with neutrophilic airway inflammation?
4. Does mucus accumulation score correlate with measured mucus volumes?

5. Do accumulation, apparent viscosity, and color scores correlate with measured viscoelasticity?
6. Is the localization of tracheal mucus related to its viscoelasticity?

Materials and methods

Definitions–In this paper, the terms "mucus (accumulation)" and "(airway) secretions" are used interchangeably to describe the gel-like mucous secretions with all their components. The horses with recurrent airway obstruction (RAO) that were used in this study consistently developed respiratory distress (increased breathing effort and rate) and cough when stabled and exposed to hay, in accordance with workshop consensus definitions [11]. Inflammatory airway disease (IAD) in this paper does not refer to the condition in young racehorses but to older "chronic coughers", in accordance with an expanded definition proposed at the International Workshop on IAD [12]. When stabled and exposed to hay, the IAD-affected horses coughed, but did not develop clinically manifest respiratory distress, while the healthy control horses showed no clinical signs. All horses in the study showed no clinical signs when at pasture and/or in low-dust indoor environment (peat moss bedding and haylage feeding). Experimental studies were approved by respective university committees for animal use of the University of Berne and of Michigan State University.

Overview of experimental protocols–Questions 1-3 (see Introduction) were addressed in the first experimental protocol, question 4 in the second, question 5 in the third, and question 6 in protocol 4.

Protocol 1: observer and horse variation and association with airway inflammation–Endoscopic examinations (Olympus[1] CF-0140L) were performed in 9

horses (2 without clinical signs, 4 with IAD and 3 with RAO; 9 to 24 years old) in the morning (0 hours), afternoon (6 hours), evening (12 hours) and on the following morning (24 hours). One day before and during the experiment, horses were not exercised, were kept in-doors bedded on shavings and were fed concentrate and/or good-quality hay. Each endoscopic examination was recorded on videotape (Sony[2] DVCAM DRS-20MDP) and video clips were digitized (DVgate Motion (acquired in .avi)- version 2.2.00 DVgate Assemble (convert to .mpeg) - version 2.2.00 [copyright Sony[2] Corp. 2000]).

Tracheo-bronchial secretion (TBS) collection was performed at 0, 6, 12 and 24 hours by introducing a teflon-coated PCV catheter through the working channel of the endoscope, instilling 10 ml of phosphate-buffered saline (PBS) through the catheter in front of the tracheal bifurcation and immediately re-aspirating the PBS mixed with secretions. From the recovered fluid, 100 and 200 μL aliquots were centrifuged for cytospin preparations. At the end of the experiments (24 hours), bronchoalveolar lavage fluid (BALF) was collected through the working channel of the endoscope, which was wedged in a peripheral bronchus [15]. Six 50-ml aliquots of PBS were infused into the tube and recovered by suction. The lavaged fluids were pooled. 100 and 200 μL aliquots were centrifuged for cytospin preparations. Total cell counts in BALF were performed manually using a hemacytometer. After staining with May-Grünwald-Giemsa, differential cell counts were obtained from TBS and BALF cytospin preparations. Differential cell counts were expressed as percent of total cells by counting 200 cells using standard morphologic criteria under a light-microscope. Absolute numbers of BALF cells were calculated from total cell counts and the proportion of each cell type.

The digitized video clips of the endoscopic examinations were viewed on personal computer screens and scored for mucus accumulation, apparent viscosity, localization and color according to the continuous scales scoring system illustrated in Fig. 1A-D. Scoring was performed in randomized order independently by three blinded observers, and repeated by each observer at least three weeks after the first scoring series.

Protocol 2: mucus accumulation scores and measured volumes of "artificial mucus"–In order to relate endoscopically scored mucus accumulation to measured volumes of mucus, "artificial mucus" made from Jell-O® (Kraft Foods[3]) was deposited with a syringe and attached catheter into a full-length excised horse trachea, which was rinsed before each procedure. The endoscope was introduced into the cranial end of the excised trachea. Increasing volumes of "artificial mucus" were deposited during continuous endoscopic observation of the trachea's full length. Deposited volumes (mL) of "artificial mucus" were recorded when the endoscopically observed accumulation corresponded to scores 0, 0.1, 1, 2, 3, 4 and 5 assessed during the endoscopic procedure by observer 1 according to Fig. 1A. A score of 0.1 was one single small, but endoscopically visible bleb of "artificial mucus". The procedure was performed three times.

Protocol 3: Mucus accumulation, apparent viscosity and color scores and measured viscoelasticity–Fifty-six endoscopic scores of mucus accumulation, viscosity and color were correlated to corresponding measurements of mucus viscoelasticity in clinically healthy and RAO-affected horses in an environmental challenge protocol. Briefly, 7 RAO-affected (9 to 26 years old) and 7 healthy control (7 to 26 years old) horses were kept at pasture and their diet was supplemented with pellets until all animals had no clinical signs of airway obstruction. Endoscopic mucus accumulation, apparent

viscosity and color scores and samples of mucus were obtained at baseline (0 hours) and 6, 24 and 48 hours after environmental challenge by stabling in stalls with straw bedding and feeding hay. Mucus was obtained from the ventral part of the trachea by means of a cytology brush passed via an endoscope. After sampling, the brush was retracted 2-3 cm into the working channel and the endoscope was withdrawn from the animal. The brush was again protruded and mucus removed from the brush to be immediately stored under light mineral oil at -80°C. The magnetic microrheometer technique was used to measure the bulk viscosity and elasticity as described [168]. Mucus accumulation, apparent viscosity and color were scored during the endoscopic procedure by observer 1 (VG) according to Fig. 1 A, C and D, respectively. Viscoelasticity data during environmental challenge can be found in Gerber *et al.* (2000 [169]). The associations of mucus accumulation, viscosity and color scores with measured viscoelasticity have not been previously reported.

Protocol 4: mucus viscoelasticity of samples from the ventral and the dorsal trachea–The relationship of the localization of mucus to its viscoelasticity was investigated by comparing viscoelasticity of samples collected ventrally to samples concurrently collected dorsally in the tracheae of six IAD-affected horses (5 to 16 years old). A dorsal and a ventral sample each were collected separately in randomized order with a clean, dry cytology brush passed via an endoscope. Mucus samples were stored at -80 C until analysis by magnetic microrheometry as outlined above and described previously in detail [168]. We report viscoelasticity (G* = vector sum of viscosity and elasticity) as log G* at 10 radian/s; dyn/cm^2.

Statistics–Statistical analyses were performed using SigmaStat for Windows, version 2.03S statistical software by SPSS[4] and NCSS[5] 6.0.22. After accepting normal distribution of scored data (Martinez-Iglewicz test for normality), data from protocol 1 were analyzed by means of a four-way ANOVA according to the model:

$$Y = \mu + H + O + HO + T + HT + OT + Error$$

where Y was the response variable (e.g., accumulation score), μ was the overall mean, H was the random effect of Horse, O was the random effect of Observer, T was the random effect of Time, HO, HT and OT were interactions of those random factors, and Error was the error due to repetition (within observer). Variance (s^2 = standard deviation^2 = σ^2) was calculated by setting the Estimated Mean Square equal to the Mean Square calculated by ANOVA, and solving for σ^2. Because there was no significant effect of time when it was considered a fixed factor, it was considered a random factor for the purpose of calculating σ^2. Between-observer variance (σ^2_O) is reported as the sum of the variances due to observer, horse*observer, and time*observer. Within-observer variance (repetition, σ^2_R) is reported as σ^2_{error}. Within-horse variance over time (σ^2_T) is reported as the sum of the variances due to time and time*horse. Between-horse (σ^2_H) variance is reported as the variance due to horse.

Coefficients of variance ([standard deviation / mean] * 100; %) were calculated for each of the 3 repeated volume measurements at mucus accumulation scores 0, 0.1, 1, 2, 3, 4, 5 in protocol 2. We report the overall mean of the coefficients of variance.

Within- and between-observer correlations for all scores (protocol 1), correlations of mucus accumulation, viscosity and color scores with mucus viscoelasticity (overall and separate by time point; protocol 3), and of mucus accumulation scores with TBS and

BALF neutrophil percentages and numbers (protocol 1) were tested with Spearman Rank Order tests. Mucus accumulation scores were correlated with TBS (sampled at 6, 12 and 24 hours) and BALF (sampled only at 24 hours) airway neutrophils in the nine horses of protocol 1. Likely due to local trauma from the repeated procedures, TBS neutrophil percentages significantly increased at 6, 12 and 24 hours compared to 0 hours (results not shown). Therefore, mucus accumulation scores were correlated with BALF neutrophil percentages and absolute numbers (at 24 hours, only time point sampled) and with TBS neutrophil percentages at 0 hours (since the values at the later time points may have been influenced by iatrogenic inflammation). Viscoelasticity of dorsal vs. ventral paired mucus samples (protocol 4) was compared by Wilcoxon Signed Ranks test. Significance limit for all tests was set at $P < 0.05$.

Results

Contributions of observer (between observer variance; σ^2_O), repetition (within observer variance; σ^2_R), time (within individual horse variance; σ^2_T) and horse (between horse variance; σ^2_H) to the total variance for mucus accumulation, viscosity, localization and color scores are presented as % of total variance (Table 1). Accumulation scores showed the lowest observer related variance (combined σ^2_O and σ^2_R), and, correspondingly, correlated best within and between observers (Table 2; Fig. 2) of all the tested scores. In contrast, observer-related variance was highest for apparent viscosity scores (combined σ^2_O and σ^2_R = 80% of total; Table 1), with complete disagreement between observer 2 and the other observers (Table 2). Mucus color and localization scores showed intermediate observer-related variance (Table 1) and correlations (Table 2; Fig. 2). Within-horse variance over time was moderate for all scores (Table 1). The range

was less than 1 score unit in most horses (Table 3) for apparent viscosity, localization and color scores, and only two of nine horses showed a range of more than 1.7 score units for accumulation scores (Table 3).

In the 9 horses, there were significant correlations between mucus accumulation score (mean of all time points, observers and repetitions) and TBS neutrophil percentage ($r^2 = 0.74$; Fig. 3 A), and BALF neutrophil percentage ($r^2 = 0.59$; Fig. 3 B) and absolute numbers ($r^2 = 0.88$; Fig. 3 C).

The experiment with artificial mucus showed that mucus accumulation scores were associated with volumes of mucus within the trachea (Fig. 4). The repeatability of this experiment was good, with a mean coefficient of variance of 31% of the 3 repetitions of 7 measurements at mucus accumulation scores 0, 0.1, 1, 2, 3, 4 and 5.

Endoscopic scores of mucus accumulation, apparent viscosity and color (n = 56; Fig. 5 A, B and C) were not significantly correlated with the corresponding measurements of mucus viscoelasticity in clinically healthy and recurrent airway obstruction (RAO)- affected horses. Conversely, a strong trend (P = 0.051) towards a significant difference was observed between viscoelasticity (log G* at 10 radian/s; dyn/cm^2) of mucus samples collected ventrally (2.0 ± 0.9) and dorsally (2.4 ± 1.1) in the trachea (n = 6 paired samples; Fig. 5 D). This difference (0.4x log units) corresponds to a 2.5-fold difference in viscoelasticity on a linear scale.

Discussion

Mucus accumulation scores were the most reliable and informative of the evaluated scores: highly reproducible within and between observers (Table 1 and 2; Fig. 2), with moderate variance over time within horses (Table 1 and 3), and related to

measured volumes of mucus (Fig. 4). The use of the illustrated scoring scale (Fig. 1 A) and endoscopic video clips, which could be watched repeatedly and in a quiet environment, likely optimized agreement within and between observers. The present results support the validity of previous findings of mucus accumulation scoring [5,46,47].

The available data [5,46,47] indicate that "no mucus or multiple small blobs" (accumulation score < 2; Fig. 1A) can be regarded as normal. Increased mucus accumulation, in contrast, is associated with coughing [5, 50, 51] and with poor racing performance [33]. Mucus accumulation scores are not specific for RAO. They can be observed in various lower airway diseases [5]. It is also interesting that in clinically healthy horses, we found no increase of accumulation scores with stabling and no correlation with airway inflammation [47].

In contrast, mucus accumulation scores correlated well with TBS neutrophil percentages and BALF neutrophil numbers in the 9 horses of protocol 1 that evenly represented the severity spectrum of non-infectious equine lower airway disease. Neutrophilic inflammation may cause mucus accumulation in horse airways through increased production and secretion of mucins [135, 170] and/or by unfavorably altering physical properties and clearability of the secretions [171, 172].

Unfortunately, measurements of viscoelasticity and other physical properties that influence clearability are technically demanding and often unavailable. Therefore, reliable endoscopic scoring systems that correlate with mucus physical properties could be useful tools for clinical and research purposes. Scores of apparent viscosity are reported to correlate with measurements of lung function [167], and were found to decrease with oral N-acetylcysteine treatment in horses with RAO in a blinded study [173].

In the present study, within-horse variance over time of mucus viscosity, localization and color scores was moderate (Table 1) and the range was less than 1 score unit in most horses (Table 3). Mucus viscosity scores did not correlate with measured viscoelasticity (Fig. 5B) and the combined within- and between-observer variance was 80% of the total (Table 1) with complete disagreement between observer 2 and the other observers (Table 2). Similarly, mucus color did not correlate with measured viscoelasticity (Fig. 5C) and, possibly due to different monitors used by the observers for scoring, showed quite high observer-related variance (Table 1 and 2). It is important to note here that other physical properties such as spinnability and adhesiveness of mucus that are also important determinants of clearability [69] were not measured or correlated with endoscopic scores in this study. Also, it may still be possible to subjectively differentiate the extremes recorded in some of the scores (Fig. 4) despite relatively high observer-related variance. Regardless of the scores assessing mucus quality, only localization is somewhat supported by the results of this study. Dorsally vs. ventrally located mucus showed a 2-fold difference of viscoelasticity (Fig. 5D), and these extremes of localization can be endoscopically differentiated (Tables 1 and 2; Fig. 2B).

In conclusion, this study showed that endoscopic scoring of mucus accumulation is a reliable clinical and research tool. Due to high observer-related variance, however, endoscopic scores of mucus viscosity, localization and color should be interpreted with caution.

Table 2-1: Variance attributable to observers (σ^2_O), repetitions (σ^2_R), time (σ^2_T), and horse (σ^2_H) for mucus accumulation, localization, apparent viscosity and color scores

		Variance ($sd^2 = \sigma^2$)				Percent of total variance			
Response Variable	**Mean**	σ^2_O	σ^2_R	σ^2_T	σ^2_H	σ^2_O	σ^2_R	σ^2_T	σ^2_H
Accumulation	2.14	0.12	0.20	0.30	1.28	6%	10%	16%	68%
Localization	2.03	0.10	0.26	0.15	0.32	12%	32%	17%	39%
Apparent viscosity	2.66	0.32	0.24	0.07	0.08	45%	34%	10%	11%
Color	3.34	0.38	0.32	0.23	0.16	35%	29%	21%	15%

Table 2-2: Spearman Rank Order correlation within and between observers

	1	2	3
Accumulation scores (0-5)			
1	0.86; 36	0.85; 36	0.77; 36
2	-	0.88; 36	0.90; 36
3	-	-	0.71; 36
Localization scores (1-5)			
1	0.27; 36	0.20; 35	0.44; 36
2	-	0.59; 35	0.34; 36
3	-	-	0.58; 36
Apparent viscosity scores (1-5)			
1	0.46; 36	0.08; 36 (NS)	0.42; 36
2	-	0.35; 35	0.03; 36 (NS)
3	-	-	0.44; 36
Color scores (1-5)			
1	0.59; 36	0.45; 35	0.52; 36
2	-	0.53; 35	0.56; 35
3	-	-	0.62; 36

Correlations are based on means of the two repetitions for each observer. Matrix: observers 1 (VG), 2 (ER) and 3 (AI). Each cell shows Spearman rank order r^2; number of valid observations. All correlations are significant, except those marked NS, not significant.

Table 2-3: Means ± SD (0, 6, 12, and 24 hours), ranges (all time points) and overall means ± SD (all time points) of all observers and repetitions for mucus accumulation, localization, apparent viscosity and color scores for each horse.

Hors	0 hours	6 hours	12 hours	24 hours	Range	Overall
Accumulation scores (0-5)						
1	0.7 ± 0.5	1.1 ± 0.4	1.6 ± 0.6	0.4 ± 0.3	0.4 - 1.6	0.9 ± 0.6
2	1.4 ± 0.8	0.7 ± 0.5	0.6 ± 0.4	0.8 ± 0.3	0.6 - 1.4	0.9 ± 0.6
3	0.9 ± 0.7	0.3 ± 0.2	2.5 ± 0.5	1.1 ± 0.4	0.3 – 2.5	1.2 ± 0.9
4	0.5 ± 0.4	2.2 ± 0.5	1.1 ± 0.6	1.2 ± 0.6	0.5 – 2.2	1.3 ± 0.8
5	4.0 ± 0.6	2.6 ± 0.5	2.8 ± 0.4	4.0 ± 0.4	2.6 – 4.0	3.3 ± 0.8
6	1.4 ± 0.7	1.9 ± 0.7	2.1 ± 0.2	1.5 ± 0.3	1.4 – 2.1	1.7 ± 0.6
7	2.8 ± 0.5	2.8 ± 0.6	2.4 ± 0.4	2.3 ± 0.6	2.3 – 2.8	2.6 ± 0.5
8	3.7 ± 0.7	3.9 ± 0.7	3.6 ± 0.7	3.5 ± 0.6	3.5 – 3.9	3.7 ± 0.6
9	3.8 ± 0.6	3.4 ± 0.5	3.5 ± 0.3	4.1 ± 0.6	3.4 – 4.1	3.7 ± 0.6
Localization scores (1-5)						
1	2.0 ± 1.3	2.8 ± 1.2	3.7 ± 1.0	1.9 ± 0.7	1.9 – 3.7	2.6 ± 1.3
2	1.8 ± 0.5	1.5 ± 0.5	1.5 ± 0.6	1.5 ± 0.4	1.5 – 1.8	1.6 ± 0.5
3	1.4 ± 0.4	1.7 ± 0.5	1.7 ± 0.4	1.9 ± 0.2	1.4 – 1.9	1.7 ± 0.4
4	1.7 ± 1.0	2.2 ± 0.4	1.9 ± 0.5	2.3 ± 0.6	1.7 – 2.3	2.0 ± 0.7
5	3.2 ± 0.9	3.3 ± 0.4	3.2 ± 0.2	3.4 ± 0.4	3.2 – 3.4	3.3 ± 0.5
6	1.2 ± 0.3	1.4 ± 0.4	1.1 ± 0.2	1.9 ± 0.7	1.1 – 1.9	1.4 ± 0.5
7	1.4 ± 0.2	1.6 ± 0.4	1.6 ± 0.5	1.9 ± 0.5	1.4 – 1.9	1.6 ± 0.4
8	1.6 ± 0.6	2.0 ± 0.4	1.5 ± 0.3	1.6 ± 0.3	1.5 – 2.0	1.7 ± 0.5
9	2.7 ± 0.6	1.9 ± 0.3	2.1 ± 0.4	3.5 ± 0.4	1.9 – 3.5	2.5 ± 0.8
Apparent viscosity scores (1-5)						
1	1.9 ± 0.8	3.0 ± 0.9	3.3 ± 0.6	2.3 ± 0.8	1.9 – 3.3	2.6 ± 0.9
2	2.5 ± 0.6	2.5 ± 0.9	2.4 ± 0.9	2.4 ± 0.7	2.4 – 2.5	2.5 ± 0.7
3	2.4 ± 0.5	2.3 ± 1.0	3.1 ± 0.8	2.8 ± 0.6	2.4 – 3.1	2.6 ± 0.8
4	3.0 ± 0.8	3.3 ± 0.4	2.9 ± 0.9	3.4 ± 0.6	2.9 – 3.4	3.2 ± 0.7
5	2.2 ± 0.7	2.2 ± 0.3	2.1 ± 0.4	2.1 ±0.2	2.1 – 2.2	2.2 ± 0.4
6	1.9 ± 0.8	2.3 ± 0.6	2.3±0.3	2.6 ± 0.7	1.9 – 2.6	2.3 ± 0.6
7	3.1 ± 0.6	2.7 ± 0.7	2.6 ± 0.7	2.6 ± 0.6	2.6 – 3.1	2.7 ± 0.6
8	2.8 ± 0.8	2.8 ± 0.7	2.9 ± 0.7	2.3 ± 0.8	2.3 – 2.9	2.7 ± 0.7
9	3.1 ± 0.7	2.8 ± 1.0	3.1 ± 0.9	3.8 ± 0.4	2.8 – 3.8	3.2 ± 0.8
Color scores (1-5)						
1	4.0 ± 0.5	3.9 ± 0.5	3.6 ± 0.4	3.7 ± 0.5	3.6 – 4.0	3.8 ± 0.5
2	4.1 ± 0.7	4.1 ± 0.4	4.2 ± 0.5	3.7 ± 0.8	3.7 – 4.2	4.0 ± 0.6
3	3.8 ± 0.6	4.3 ± 0.6	1.6 ± 0.4	3.5 ± 1.0	1.6 – 4.3	3.3 ± 1.2
4	2.6 ± 0.6	2.5 ± 0.6	2.4 ± 0.9	2.5 ± 0.5	2.4 – 2.6	2.5 ± 0.6
5	3.3 ± 0.4	3.4 ± 0.5	3.5 ± 0.5	3.5 ± 0.4	3.3 – 3.5	3.4 ± 0.5
6	3.6 ± .1.1	3.8 ±0.5	3.5 ± 0.4	3.2±0.9	3.2 – 3.8	3.5 ± 0.7
7	2.6 ± 0.7	2.9 ± 0.8	3.6 ± 1.0	3.1 ± 0.6	2.6 – 3.6	3.0 ± 0.8
8	4.2 ± 0.3	3.3 ± 1.0	3.4 ± 0.9	3.7 ± 0.6	3.3 – 4.2	3.7 ± 0.8
9	2.5 ± 0.6	2.4 ± 1.1	2.2 ± 1.0	2.3 ± 0.9	2.2 – 2.5	2.4 ± 0.9

Fig. 2-1: Scoring system for mucus accumulation (A), localization (B), apparent viscosity (C) and color (D).

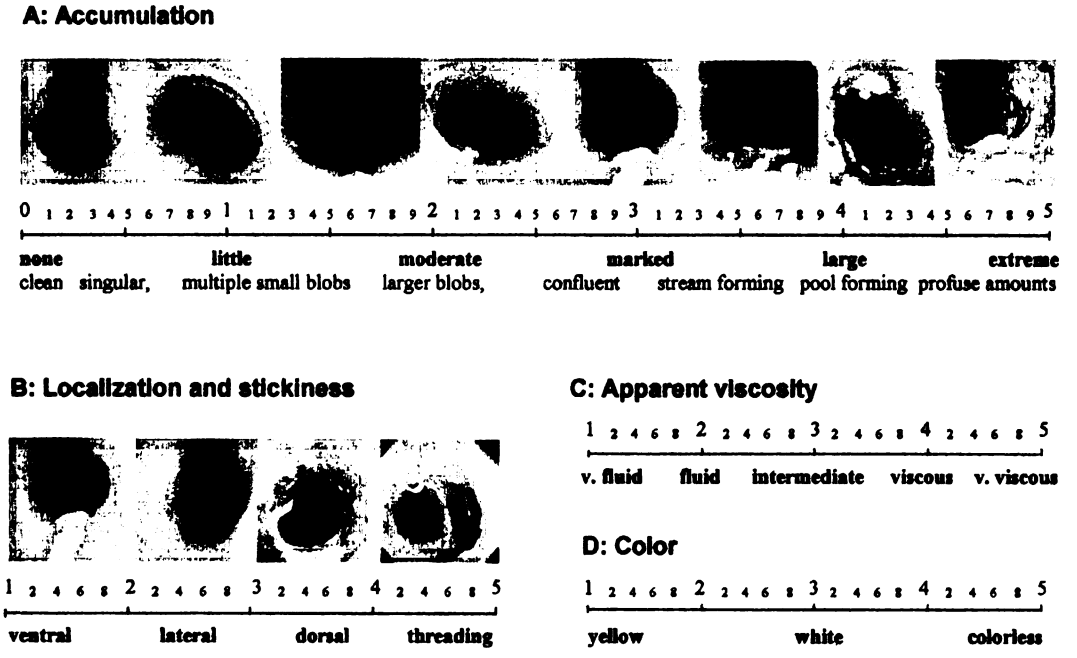


Fig. 2-2: Intraobserver (observer 1, round 1 and 2) correlations for mucus accumulation (A), localization (B), apparent viscosity (C) and color (D) scores.

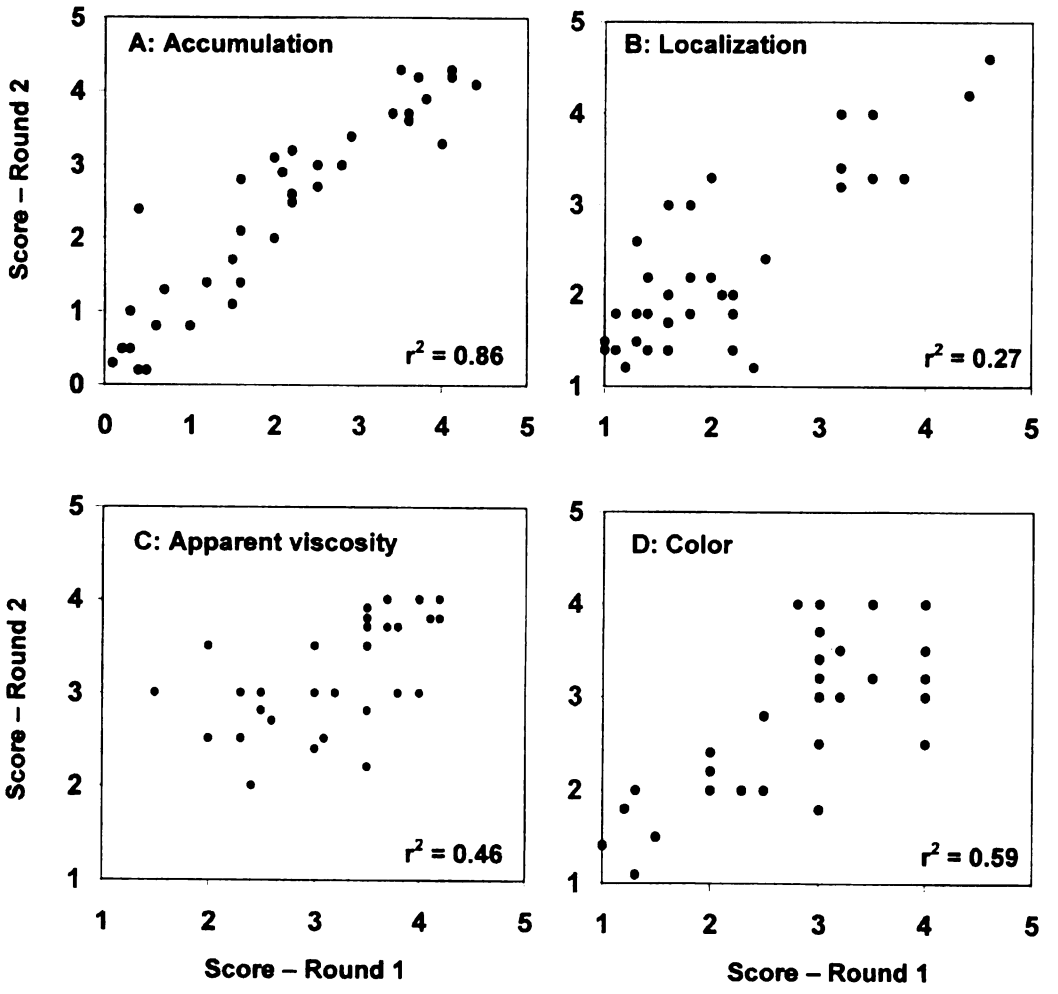


Fig. 2-3: Associations of mucus accumulation scores with tracheo-bronchial secretion (TBS) neutrophil percentages (A) at 0 hours, and with bronchoalveolar lavage fluid (BALF) neutrophil percentages (B) and BALF neutrophil absolute numbers (C) at 24 hours.

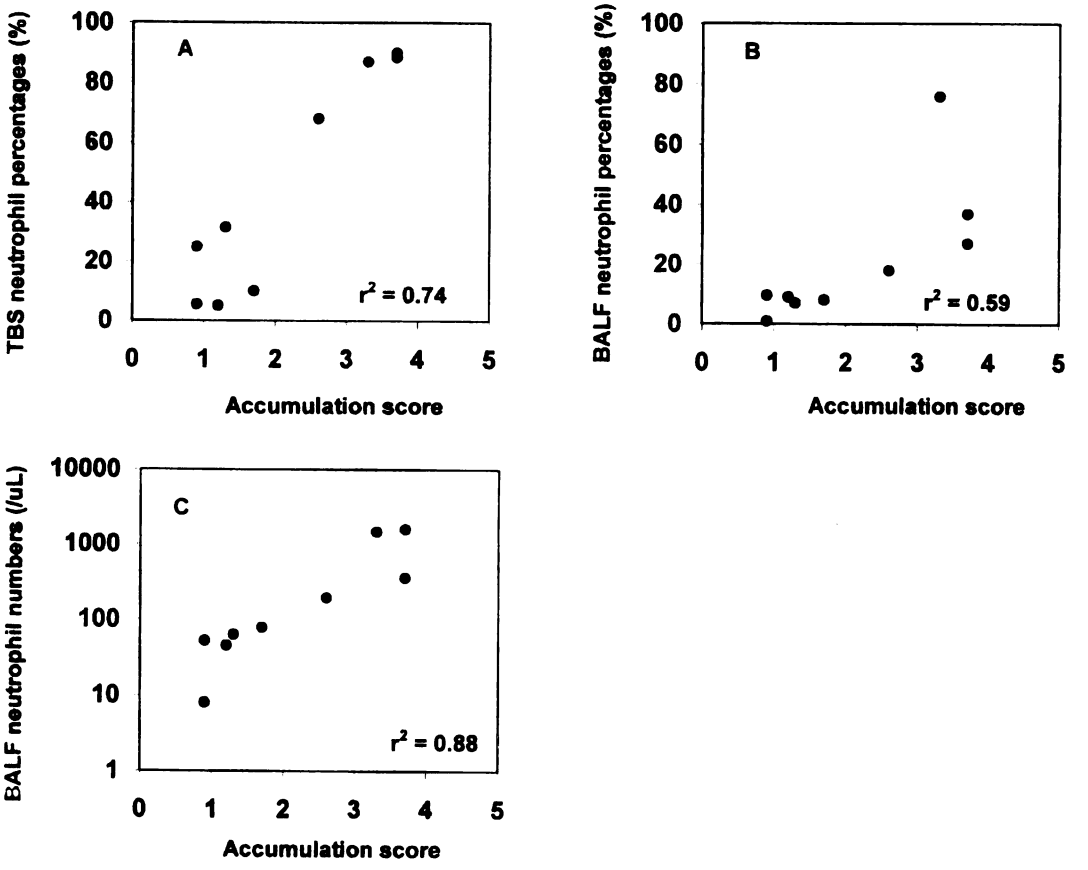


Fig. 2-4: Relationship between mucus accumulation score and volumes of "artificial mucus" (Jell-O®[1]).

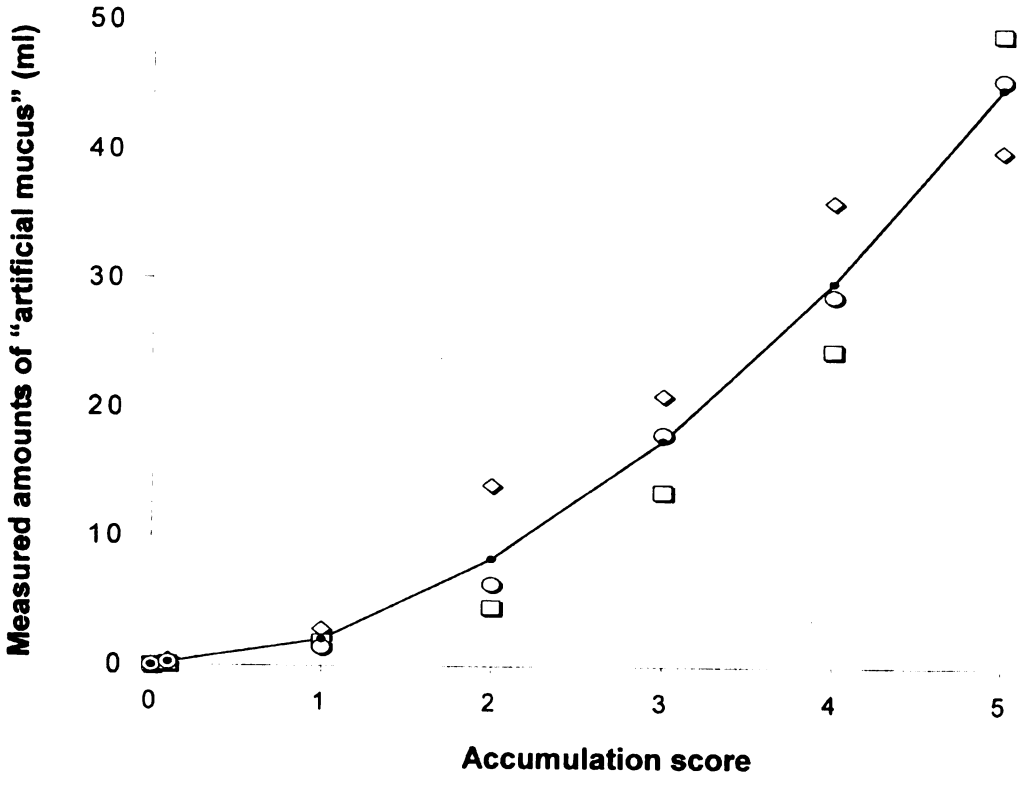
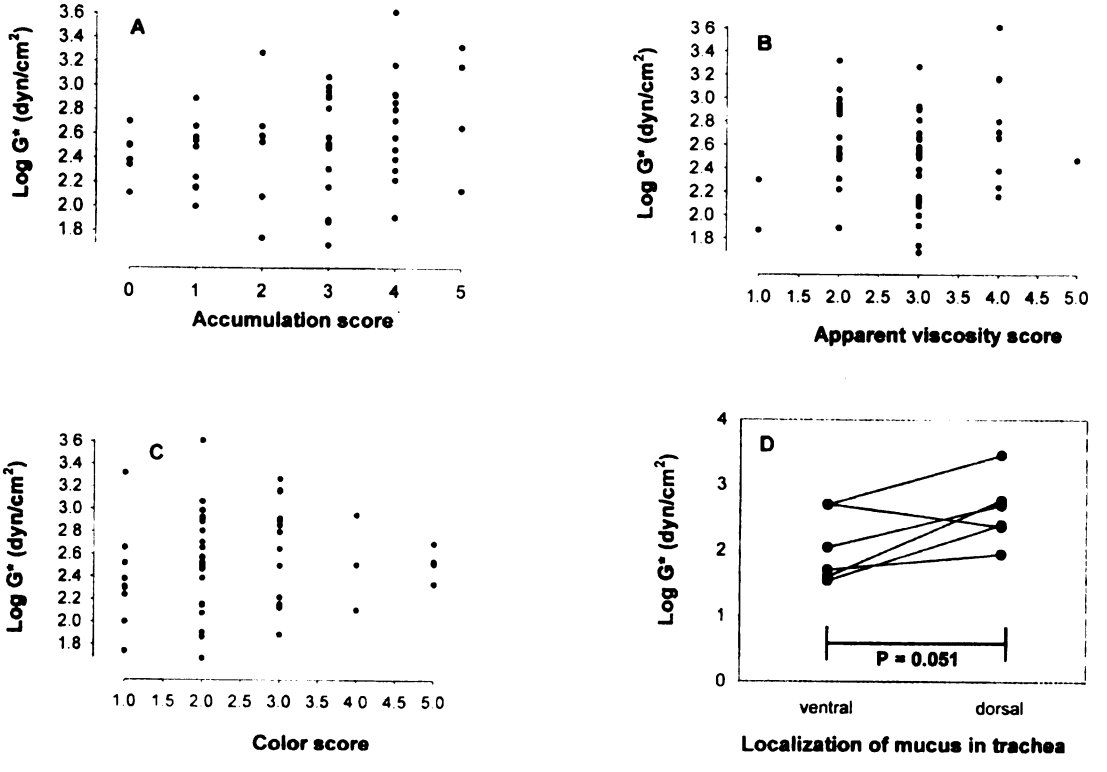


Fig. 2-5: Relationships between mucus accumulation (A), apparent viscosity (B) and color (C) scores and measured viscoelasticity (log G* at 10 radian/s; dyn/cm^2) and trend (P = 0.051) towards a significant difference between viscoelasticity (D) of mucus samples collected ventrally vs. samples collected dorsally in the trachea at the same time.



CHAPTER 3

AIRWAY MUCUS ACCUMULATION–COMPARISON OF RAO-AFFECTED AND CLINICALLY HEALTHY CONTROL HORSES BEFORE AND DURING ENVIRONMENTAL CHALLENGE

Gerber, V., Lindberg, A., Berney, C., Robinson, N.E. Airway mucus in RAO – short-term response to environmental challenge. (2003). Accepted for publication in *J Vet Int Med.*

In the previous study (chapter 1) I showed that endoscopic scoring is a reliable tool to quantify airway mucus accumulation in horses.

In order to test the hypothesis that airway mucus accumulation is a function of disease status and/or environment, I compared mucus accumulation scores in RAO-affected and clinically healthy control horses before (when they were at pasture) and after (6, 24 and 48 hours) stabling on straw bedding and hay feeding.

Several alternative outcomes were possible: No difference of mucus accumulation scores between RAO-affected and control horses and no difference before and after stabling. Significant effects of disease group (i.e., difference between RAO and controls) and/or environment (i.e., difference before and after stabling); significant interaction effects of disease group and environment (i.e., change in mucus accumulation with stabling in one group but not the other).

Abstract

Mucus accumulation and neutrophilic inflammation in the airways are hallmarks of RAO. Endoscopically visible mucus accumulations, however, have not been studied during exposure to dusty hay and allergens (i.e., environmental challenge). We hypothesized that a) RAO-affected horses have increased mucus accumulation compared to controls, b) mucus accumulations increase in RAO-affected horses during environmental challenge, and c) environmental challenge also induces neutrophilic inflammation and mucus accumulation in control horses. Mucus accumulation was graded endoscopically (mucus grades [MG] 1-5) and airway inflammation was evaluated by bronchoalveolar lavage fluid (BALF) cytology before (0 hours) and during (6, 24, 48 hours) environmental challenge. Large amounts of mucus (MG 4-5) were specific for RAO-affected horses in this study. Variation among controls was considerable, however, and intermediate grades (MG 2-3) were non-specific, showing complete overlap between the 2 groups. Mucus accumulations [median (25^{th}, 75^{th} percentile)] increased in RAO-affected horses from 2.5 (1.5, 3.5) MG at baseline to 3.5 (2.0, 4.0), 4.0 (3.0, 4.0) and 4.0 (4.0, 4.0) MG at 6, 24, and 48 hours, respectively. MG did not increase in controls, overall 1.0 (1.0, 2.0) MG, even though controls also showed a moderate increase of BALF neutrophils. In conclusion, mucus accumulations, before and especially after exposure to dust and allergens, are increased in RAO-affected horses compared to controls. Healthy controls show considerable variability in mucus accumulation, but despite an influx of neutrophils into the airways, no increase of mucus accumulation after exposure to hay dust.

Introduction

Mucus accumulation and neutrophilic inflammation in the airways are hallmarks of recurrent airway obstruction (RAO) [3]. The changes in BALF cytology in response to stabling and hay feeding (i.e., environmental challenge) in RAO have been well characterized [15]. Most prominent is neutrophilic inflammation, and neutrophils accumulate in the lung within 7 hours of environmental challenge [14]. Increases in BALF neutrophils during environmental challenge, however, are not limited to RAO horses, but also have been reported in clinically healthy animals [47, 174]. In other species, airway inflammation, especially neutrophils and their products, can cause increased mucin gene expression and mucus hypersecretion [135, 150, 175, 176] as well as unfavorable changes in mucus rheology [171, 177]. Changes in mucus rheology may lead to decreased clearance of secretions as reported in RAO [58, 64]. Both increased production and decreased clearance can lead to mucus accumulation in the airways. To date, however, the amount of endoscopically visible mucus accumulations in airways before and during environmental challenge has not been investigated in horses. We hypothesized that RAO-affected horses have increased mucus accumulation compared to controls and that mucus accumulations increase during environmental challenge. We further hypothesized that environmental challenge also induces neutrophilic airway inflammation and mucus accumulation in clinically healthy control horses. This study investigates changes in mucus accumulation and BALF cytology in RAO-affected and control horses before (0 hours) and during (6, 24, 48 hours) environmental challenge.

Materials and methods

Animals and study design–The protocol was approved by the All-University Committee for Animal Use and Care of Michigan State University. Twelve horses (6 mares and 6 geldings), 9 to 25 years old (18.1 ± 5.2 years, mean ± SD), with a previous diagnosis of RAO and manifesting respiratory distress during environmental challenge served as the RAO group. Eleven horses (4 mares and 7 geldings) aged 7 to 27 years (15.2 ± 7.9 years, mean ± SD) and without clinical signs of respiratory tract disease before and during environmental challenge, served as the control group. The horses in both groups were selected and monitored according to criteria based on history and on numerically graded clinical signs of respiratory distress as previously described [178]. Both groups were drawn from closed research herds in the Department of Large Animal Clinical Sciences, Michigan State University that develop (RAO group) or do not develop (control group) signs of obstructive pulmonary disease when stabled and fed hay. All horses were vaccinated against equine influenza, rhinopneumonitis, and Eastern and Western equine encephalitis. Horses were dewormed within 2 months of the study. The study was performed between the months of August and October. Before entering the study, the horses were kept at pasture and their diet was supplemented with pellets when grass was sparse until all animals showed no clinical signs of airway obstruction. Horses were transported to the laboratory in an open-sided stock trailer for less than 10 minutes and without their heads tied. Clinical assessment, endoscopic mucus grades (MG) and BALF were obtained at baseline (0 hours) and after 6, 24, and 48 hours of environmental challenge by stabling in stalls with straw bedding and feeding of dusty hay.

Bronchoalveolar lavage fluid (BALF) sampling and endoscopic mucus scoring–BALF was collected, processed and analyzed according to guidelines set forth in the International Workshop on RAO [11]. Briefly, the endoscope (Olympus GIF 300, custom made) was directed to a different site within the lung for each collection (0, 6, 24, and 48 hours). After the endoscope was wedged in an airway, 3 100-ml aliquots of phosphate-buffered saline were instilled into the airway and then aspirated. The aspirates were pooled and mixed. Total cells in BALF were counted by use of a hemacytometer. Differential cell counts were made on 200 cells of a cytocentrifuge preparation stained with Diff-Quick (Dade Bering) and performed by one observer (Lindberg). Total neutrophil counts were calculated from total and differential cell counts.

The amount of mucus visible in the trachea was graded on a validated endoscopic scale of 0-5 MG [179]: 0 (no visible mucus), 1 (singular small blobs), 2 (multiple blobs only partly confluent), 3 (mucus ventrally confluent), 4 (large ventral pool), 5 (profuse amounts of mucus occupying more than 25% of tracheal lumen). The entire trachea was assessed and gradings were determined according to the largest amount observed in any part of the trachea, which usually was the most caudal aspect cranial to the bifurcation. Two observers (VG and AL) graded MG in 7 RAO-affected horses and 6 controls and in 5 RAO-affected horses and 5 controls, respectively.

Statistical analysis–All statistical analyses were performed using the SAS System for Windows, version 8.02. MG (dependent measure of interest) and were analyzed by non-parametric repeated measures analysis of longitudinal data (SAS-macros). Independent factors included 2 levels each of the between subjects factors, observer (VG vs. AL) and group (control vs. RAO horses), and 4 levels (0, 6, 24, and 48 hours) of the

repeated measure, time. Subanalyses were performed using Wilcoxon-sign-ranks test (for paired comparison within groups over time) and Kruskal-Wallis (Wilcoxon-rank-sum; for non-paired comparison between groups) when main effect, interaction effect, or both were significant. BALF total cell counts and differential cell percentages and numbers (total cell count × percentage of each cell type) did not satisfy the model assumptions of equal variance and normal distribution for parametric analysis and therefore were analyzed like the MG data, except that there was no observer factor. Sensitivity [true positive / (false negative + true positive)] and specificity [true negative / (false positive + true negative)] of low MG values (<2) for clinically healthy horses and of high MG values (>3) for RAO-affected animals were calculated. All data were expressed as medians and quartiles (i.e., 25^{th}, 50^{th} and 75^{th} percentiles). Significance limit was set at $P < 0.05$.

Results

Endoscopic MG–A complete set of observations (n = 92) of MG was obtained and summary data are presented in Table 1. Mucus accumulations over time also are illustrated in Fig. 1A, and the overall distributions in both groups are shown as histograms in Fig. 2. There was no effect of the observer (VG vs. AL), but a significant difference in MG between control and RAO-affected horses (main group effect), as well as significant time and interaction effects. Subanalysis determined that MG were different between groups at time points 6, 24 and 48 hours, and showed a strong trend (P = 0.054) for a difference at baseline (0 hours). MG significantly increased over time in RAO (6, 24, and 48 hours differed from baseline), but not in control horses. Overall (Fig. 2),

observations of "no mucus or a few single mucus droplets" (MG 0-1) had high specificity (0.92), but low sensitivity (0.52) for clinically healthy controls. Large amounts of mucus (MG 4-5) also showed high specificity (0.98), but low sensitivity (0.56) for RAO. In contrast, intermediate MG ("multiple only partly confluent blobs to ventrally confluent mucus"; MG 2-3) completely overlapped between clinically healthy and RAO-affected horses.

BALF cytology changes and relationship with tracheal mucus grades–A complete set of BALF samples (n = 92) was available for cytological evaluation. Summary data of BALF cytology (total cell counts and differential cell percentages) are presented in Table 1. BALF differential cell percentages, total cell counts and differential cell numbers (total cell count × percentage of each cell type) were analyzed statistically. There was a significant effect of group, time, and interaction on BALF lymphocyte, neutrophil, macrophage, mast cell, and epithelial cell percentages, respectively. Results of differential cell percentages subanalysis are presented in Table 1. There was a significant effect of group, time and interaction on BALF total cell counts and on neutrophil cell counts. Subanalysis determined that total cell counts and neutrophil cell counts were different between groups at time points 6, 24, and 48 hours, but not at baseline (0 hours). Total cell counts increased significantly over time in RAO (6, 24, and 48 hours differed from baseline), but not in control horses. Fig. 1B illustrates how neutrophil numbers increased significantly over time in RAO (6, 24, and 48 hours differed from baseline) and also in control horses (24 and 48 hours differed from baseline). Further, there was a significant effect of time on BALF epithelial cell numbers, due to a decrease of epithelial cell numbers at 24 hours compared to baseline in control

horses. No main or interaction effects were observed for lymphocyte, eosinophil, mast cell, and macrophage numbers.

Discussion

RAO-affected horses had more mucus accumulation at all times compared to control horses (Fig. 1A). Even when at pasture, removed from allergen exposure and in clinical remission, RAO-affected horses showed a strong trend for increased mucus accumulation. We have previously demonstrated persistent alterations of mucin-side-chain-associated carbohydrates (increased concentrations of α1,2-fucose) in BALF during RAO-remission [54]. These complementary findings suggest that in RAO the mucus apparatus undergoes changes that persist even during clinical remission when clinical signs, bronchospasm, and inflammation have waned or are undetectable. Within 6 hours of exposure to the stable environment, however, mucus accumulation further increased in RAO-affected animals (Fig. 1A). Overall (all time points; Fig. 2), we noted that none of the RAO-affected horses were without visible mucus at any time, and only 8% of the observations showed just singular small blobs. More than two thirds (77%) of the endoscopic evaluations showed completely confluent secretions or more mucus (MG 3-5) with large ventral pools seen in almost half of the observations. In contrast, only 1 control horse had a large ventral pool and none showed profuse amounts of mucus occupying more than 25% of the tracheal lumen. Still, almost half of the observations in controls (21 of 44; 48%) showed more than a few blobs of mucus (MG > 1). This demonstrates that although controls had less mucus accumulation than RAO-affected animals, the amount of tracheal secretions can vary much more in clinically healthy

horses than previously reported [5]. Our results, however, did not support the hypothesis that mucus accumulation would increase with stabling in control horses. No change in mean MG was observed over time (Fig. 1A).

The distribution of mucus accumulation in both groups (Fig. 2) thus showed that "no mucus or a few single mucus droplets" in the trachea (MG 0-1) rarely is seen in RAO horses and can be regarded as normal. Large amounts of mucus (MG 4-5), on the other hand, had a high specificity for RAO in the 2 groups we studied. Intermediate MG ("multiple only partly confluent blobs to ventrally confluent mucus"; MG 2-3), however, were a non-specific finding, for which clinically healthy and RAO-affected horses completely overlapped.

At baseline, BALF neutrophil percentages were not significantly different in the RAO group, 4.3 (2.4, 15.1) as compared to controls, 5.0 (1.6, 6.0). The 75^{th} percentile shows, however, that increased neutrophil percentages were present in some RAO-affected horses at baseline, even though all horses were in clinical remission when they entered the study. Within 6 hours of environmental challenge, neutrophils increased to over 100 per uL in RAO-affected horses, and remained at those concentrations by 24 and 48 hours after stabling. In control horses, BALF neutrophils also increased during environmental challenge (Fig. 1B, Table 1). Similar degrees of neutrophilic inflammation in response to stabling have been described previously in clinically healthy horses [47, 180]. When compared to normal values proposed by the International Workshop on IAD [12], in all horses, one or more variables (visible mucus accumulation, percent neutrophils, eosinophils or mast cells; means in Table 1) over time could be considered abnormally increased, and thus signs of IAD. Also, mast cell, lymphocyte and

macrophage percentages (but not absolute numbers) significantly decreased in RAO horses after stabling (Table 1). This finding likely represented a relative change due to the large increase in neutrophils in this group. There was no evidence that the increases "above normal values" and relative changes in these cell types were related to RAO or parasitism. IAD is as yet an ill-defined syndrome, and it seems cautious not to over-interpret our findings, especially because neutrophilic inflammation in controls could have been caused partly by the repeated procedures. Nevertheless, the present results agree with those of previous studies [9, 10, 47, 180] that indicate that subclinical levels of IAD are present in a majority of stabled horses. The significance of these subclinical levels of IAD, which may be a "normal" (i.e., common) reaction to the high dust loads that horses are exposed to in a conventional stable environment [6, 8], warrants further investigation.

The repeated BAL procedures potentially could have influenced not only BALF cytology but also MG. Possible influences could be "washing out" of mucus, iatrogenic inflammation caused by the BAL procedures and, because the observers were not blinded to the diagnoses of the horses, biased MG. However, MG in the present study agree with those observed when mucus was graded by a blinded observer in 6 control and 6 RAO horses before (1.8 ± 0.9 and 2.2 ± 1.1, respectively) and after 3 days (1.6 ± 1.1 and 3.5 ± 7.6, respectively) of stabling, without any invasive procedure between these measurements (Robinson, unpublished data).

In conclusion, both mucus accumulations and BALF neutrophils increase in RAO-affected horses and are considerably higher than in control horses. The distribution of mucus accumulation in this study (Fig. 2) suggests that "no mucus or a few single

mucus droplets" in the trachea (MG 0-1) can be regarded as normal. Large amounts of mucus (MG 4-5), on the other hand, were specific for RAO in this study, but could certainly also be caused by other airway diseases in a random population of horses. Clearly, intermediate amounts of mucus ("multiple only partly confluent blobs to ventrally confluent mucus"; MG 2-3) were a non-specific finding, for which clinically healthy and RAO-affected horses completely overlapped. Persistent up-regulation of mucus production in remission and increased accumulation of secretions in exacerbation (likely due to decreased clearance and increased secretion) appear to characterize the mucus apparatus in RAO.

Table 3-1: Mucus grades and bronchoalveolar lavage fluid cytology in RAO-affected horses and control horses before (0 hours) and during (6, 24, 48 hours) environmental challenge: medians with quartiles (25^{th}, 75^{th} percentiles) for mucus grade (MG), total cell count (tcc; x cells/μl), percentages of lymphocytes (%lym), macrophages (%mac), neutrophils (%neu), eosinophils (%eos), mast cells (%mas), and epithelial cells (%epi).

	RAO-affected horses (n = 12)			
hours	0	6	24	48
MG	2.5 (1.5, 3.5)$^{+}$	3.5 (2.0, 4.0)*#	4.0 (3.0, 4.0)*#	4.0 (4.0, 4.0)*#
tcc	73.2 (39.8, 123.8)	210.0 (130.0,	260.8 (161.7,	176.5 (119.5,
%lym	48.5 (36.6, 64.8)	14.1 (10.8, 36.2)*#	14.3 (8.3, 27.8)*#	10.8 (4.2, 19.3)*#
%mac	29.9 (22.3, 39.3)	10.4 (6.3, 16.5)*#	9.3 (4.8, 13.0)*#	14.5 (6.3, 20.3)*#
%neu	4.3 (2.4, 15.1)	75.1 (21.8, 87.6)*#	75.3 (56.3, 85.0)*#	69.5 (55.5, 81.5)*#
%eos	0.0 (0.0, 0.2)	0.0 (0.0, 0.5)	0.0 (0.0, 0.3)	0.0 (0.0, 0.5)
%mas	5.2 (3.0, 7.0)	1.5 (0.6, 4.0)*#	0.9 (0.3, 3.0)*	1.0 (0.0, 6.0)
%epi	0.5 (0.0, 1.7)	0.0 (0.0, 2.5)	0.0 (0.0, 0.2)*	0.0 (0.0, 1.7)
	Control horses (n = 11)			
MG	1.0 (1.0, 2.0)	2.0 (1.0, 2.8)	2.0 (1.0, 2.8)	1.0 (1.0, 2.0)
tcc	61.0 (20.3, 95.6)	41.7 (19.4, 95.6)	67.5 (27.9, 118.1)	95.0 (23.9, 96.9)
%lym	42.0 (35.5, 53.2)	52.0 (36.9, 57.9)	46.5 (35.0, 50.4)	47.0 (31.6, 54.7)
%mac	35.0 (28.6, 43.7)	23.9 (17.3, 34.6)	28.0 (19.2, 36.9)	27.0 (20.7, 37.6)
%neu	5.0 (1.6, 6.0)	9.0 (3.9, 19.5)*	16.2 (7.0, 30.7)*	18.0 (15.0, 27.5)*
%eos	0.0 (0.0, 0.93)	0.2 (0.0, 0.2)	1.0 (0.2, 2.6)	0.5 (0.0, 1.0)
%mas	5.8 (2.5, 11.5)	5.5 (4.1, 7.1)	2.2 (1.0, 8.4)*	3.0 (2.6, 10.3)
%epi	1.5 (1.0, 5.3)	0.5 (0.1, 1.9)*	0.0 (0.0, 0.9)*	0.5 (0.0, 0.9)*

*Significantly different ($P < 0.05$) from baseline (0 hours); #significantly different ($P < 0.05$) from control at same time point; $^{+}$trend toward a difference compared with control at that time point ($P = 0.054$).

Fig. 3-1: Boxplots of mucus grades (A) and bronchoalveolar lavage fluid neutrophil numbers (B) in recurrent airway obstruction (RAO)-affected (grey boxes) and control horses (white boxes) before and 6, 24, and 48 hours after stabling. *Significantly different ($P < 0.05$) from 0 hours. #Significantly different from control at same time point; $^{+}$trend toward a difference compared to control at that time point ($P = 0.054$).

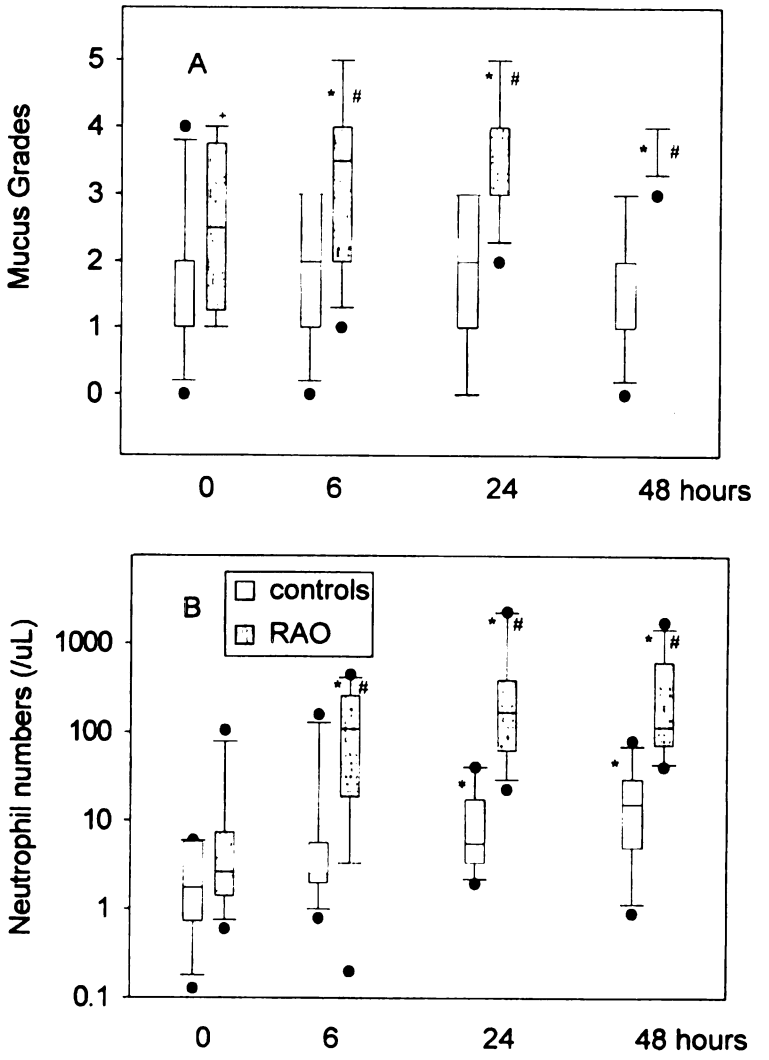
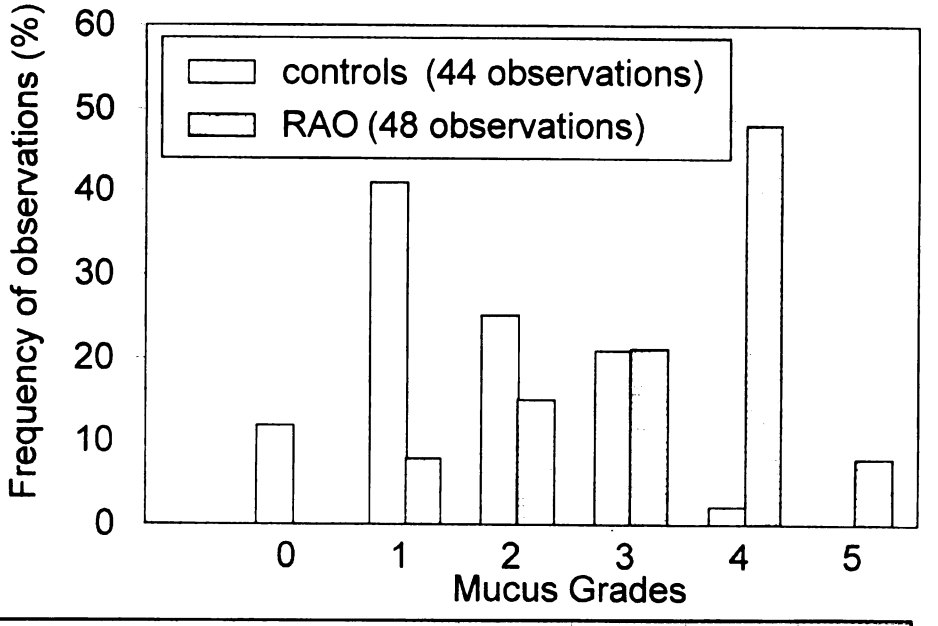


Fig. 3-2: Distribution histogram of mucus grades (MGs). Graph shows relative frequency (%) of each MG (0-5) in controls (clinically healthy horses; white columns) and in recurrent airway obstruction (RAO)-affected animals (gray columns). Numbers of observations are listed in the attached table. There was a significant difference in MGs between control and RAO-affected horses (main group effect). MG 0-1 had a good specificity (0.92), but low sensitivity (0.5) for controls. Large amounts of mucus (MG 4-5), on the other hand, showed a high specificity (0.98), but also low sensitivity (0.56) for RAO. The intermediate MGs 2-3 were a non-specific finding, where clinically healthy and RAO-affected horses completely overlap.



		0	1	2	3	4	5
number of observations	controls	5	18	11	9	1	0
	RAO	0	4	7	10	23	4

CHAPTER 4

COMPARISON OF AIRWAY MUCUS ACCUMULATION IN TWO AGE GROUPS OF CLINICALLY HEALTHY WELL-PERFORMING SPORT HORSES IN A CONTROLLED ENVIRONMENT

Gerber, V., Robinson, N.E., Luethi, S., Marti, E., Wampfler, B., Straub, R. Airway inflammation and mucus in two age groups of asymptomatic well-performing sport horses. (2003). *Equine Vet. Journal*, **35**, 491-495.

The previous study (chapter 3) showed that airway mucus accumulation is a function of disease status, i.e., mucus was increased in recurrent airway obstruction (RAO)-affected horses overall. In addition, an interaction of disease status and environment was observed, i.e., mucus increased with exposure to hay and straw in RAO-affected, but not in clinically healthy horses.

In order to test the hypothesis that airway mucus accumulation is also a function of age, I compared mucus accumulation scores in two age groups of clinically healthy well-performing sport horses that lived in the same controlled and constant stall environment.

Several alternative outcomes were possible: no difference between age-groups; increased mucus in the older horses, as suggested by research in human smokers that produce more sputum with increasing pack-years; or, increased mucus in younger horses, as observed in racehorses entering training facilities.

Abstract

Mucus quantity and quality and BALF differential cytology were investigated in thirteen younger horses (Y) with a mean age of 5 years and 13 older horses (O) with a mean age of 15 years, without historical or clinical evidence of lower airway disease. The only differences between the age groups were increased BALF total and lymphocyte cell counts in the younger horses. We found that clinically healthy older horses, having been exposed 10 more years to conventional stable environment without developing clinical signs of lower airway disease, do not show increased subclinical airway inflammation or mucus accumulation. Overall, however, large interindividual variations were observed in mucus quantity scores as well as BALF neutrophil, eosinophil, mast and epithelial cells, and the ranges and means of the variables were larger than previously described as normal. If inflammatory airway disease (IAD) is simplistically defined as >10% BALF neutrophils and/or more than just a few blobs of mucus in the trachea, then the majority of these asymptomatic horses were suffering from subclinical IAD. Why, then, was no negative effect noted on the present and past performance in any of the sport horses we examined? We propose that mild to moderate degrees of airway inflammation and mucus accumulation are a normal (i.e., observed in the majority of individuals) reaction to conventional stable environment, and that the significance of such findings should be interpreted according to the level of respiratory performance expected from an individual. Moreover, a definition of IAD based on airway inflammation and mucus alone without a measure of airway obstruction and hyperreactivity may be inadequate for clinical purposes.

Introduction

Horses housed permanently indoors, bedded on straw and fed hay (conventional stable environment) show high incidences of chronic obstructive pulmonary disease (COPD), 54% [181] and even 79% [10]. However, the term "COPD" as used in these studies may encompass very variable degrees of lower airway disease. The common denominators are airway inflammation and mucus accumulations. Recently [11, 12], more rigid diagnostic criteria have been proposed to differentiate the severe airway disease known as recurrent airway obstruction (RAO) from milder forms of airway inflammation. Briefly, the presence of recurrent and reversible airway obstruction distinguishes RAO from inflammatory airway disease (IAD), which is characterized by airway inflammation, mucus accumulation and hyperreactivity in the absence of dyspnea.

IAD, even in the absence of clinical signs such as cough or abnormal lung sounds, may negatively affect racing performance [33]. Most studies on IAD have focused on young racehorses. However, the above-cited surveys [10, 181] suggest that milder degrees of airway disease are also common among stabled sport horses of various ages. It has been proposed, but not shown, that subclinical degrees of IAD may affect the performance of sport horses. We chose to test this hypothesis by determining what degrees of airway inflammation and mucus occur in asymptomatic horses performing well in dressage and show jumping.

The causes and pathogenesis of IAD are incompletely understood and may include inhaled irritants as well as infectious agents [53]. There is evidence, that bringing asymptomatic horses of varying age groups from pasture into a conventional stable environment causes acute neutrophilic airway inflammation in asymptomatic horses [47,

180]. In contrast, we showed that mucus accumulations, even though they vary considerably between asymptomatic horses, do not seem to increase after short-term stabling [182]. There is no information on the effects of long-term exposure, however. In smokers, the duration of exposure (i.e., pack-years) to inhaled irritants (i.e., cigarette smoke causing neutrophilic airway inflammation) correlates with mucus accumulation and viscosity [183]. We hypothesized that similar changes may occur in horses that had been stabled for years and that even though older horses were asymptomatic, they would have increased degrees of subclinical lower airway disease, and that mucus accumulation would be related to neutrophil numbers in the airways. .

We tested the above hypotheses in two age groups of clinically and historically healthy sport horses that were performing well. All the horses were housed in the same conventional stable environment. The present study 1) investigated what degrees of airway inflammation and mucus are present in asymptomatic well-performing sport horses, 2) examined the relationship between airway inflammation and mucus, and 3) compared older to younger individuals.

Materials and methods

Stable environment and management conditions of horses–The Swiss National Equine Centre is stocked with over 200 Warmblood horses. They typically enter the facility at 2-4 years of age, and thereafter live permanently in a conventional stable environment: Horses are bedded on straw and housed 20-22 hours per day in box stalls or tie stalls. All horses are used daily (except for one resting day per week) for dressage and/or show jumping, most of them compete regularly. A combination of good quality

hay and haylage is fed three times per day along with individual rations of concentrated feed. Primary health care is provided at the Swiss National Equine Centre, all medical records are kept with the resident veterinarian.

Selection of horses and design of the study–Initially, 16 horses from among those younger than 8 years of age and 18 from among those older than 12 years were randomly chosen. Predetermined exclusion criteria were then applied to eliminate horses with historical and/or clinical evidence of lower airway disease: Historical criteria for exclusion were based on medical records and included a past history of chronic lower airway disease for more than three months, and/or infectious lower airway disease in the previous three months. Clinical exclusion criteria included evidence of lower airway disease such as cough, mucoid nasal discharge and/or abnormal lung sounds and/or evidence of infection. Clinical signs were evaluated before, during (coughing) and immediately after an exercise test consisting of walk, trot and canter for 10 minutes each (examined by Straub). Evidence of infection was provided by physical examination as well as complete blood count and serum chemistry profile. Four horses older than 12 years of age were eliminated because of a history of chronic lower airway disease, and 1 horse younger than 8 years had suffered from recent infectious lower airway disease. A further 3 horses (2 older than 12 years of age, 1 younger than 8 years) were eliminated from the study because of clinical signs of lower airway disease. Thirteen younger horses (younger group; Y), 4 fillies and 9 geldings, with a mean age of 5 years (±1, SD) and 13 older horses (older group; O), all geldings, with a mean age of 15 (±2, SD) qualified for the study (Table 1).

Bronchoalveolar lavage fluid (BALF) sampling and analysis–BALF was collected by means of a 197-cm endoscope (CF-VL Video colonoscope, Olympus[1]) that was passed via the nares and wedged in a peripheral bronchus. Six 50-mL aliquots of phosphate buffered saline (PBS) were infused into the tube and recovered by suction. The lavaged fluids were pooled and the total recovered volume determined. Total cell counts in BALF were performed manually using a hemacytometer. Cell smears were made with a cytocentrifuge and stained with Diff-Quick. Differential cell counts of lymphocytes, macrophages, neutrophils, eosinophils, mast cells and epithelial cells were performed by counting 200 to 240 cells per sample.

Mucus scores–The amount of mucus visible in the trachea (mucus quantity scores) was graded on a scale of 0 (no visible mucus), 1 (singular small blobs), 2 (multiple blobs only partly confluent), 3 (mucus ventrally confluent), 4 (large ventral pool) to 5 (profuse amounts of mucus occupying more than ¼ of tracheal lumen). We have previously shown that these mucus accumulation scores are reproducible within and between observers, and within horses and correlate with measured amounts [179]. One observer (Gerber) graded all mucus scores.

Statistical analysis–Statistical analyses were performed using SigmaStat for Windows, version 2.03S statistical software by SPSS[2]. Absolute numbers of BALF cells were calculated from total cell counts and the proportion of each cell type. Grouped data were described with mean, standard deviation (SD), and coefficient of variation (CV). All data were analyzed using non-parametric tests. Mann-Whitney Rank Sum Tests were used for comparisons between age groups. Spearman Rank Order correlations were used to test for associations of age with mucus scores and BALF cytology parameters.

Correlations of mucus scores with BALF neutrophil percentages and numbers were also tested with Spearman Ranks Order correlations. Significance limit was set at $P < 0.05$.

Results

Interindividual variability–Complete sets of observations of mucus scores as well as of BALF cytological variables are shown in Table 1. The variation (CV) of mucus scores was high overall (65.3%; all individuals combined) and in either age group (Table 1; illustrated in Fig. 1A). The CVs of BALF cytological parameters overall (all individuals) were 37.7% for total cell counts, 24.6% for lymphocytes, 21.5% for macrophages, 86.5% for neutrophils, 392.1% for eosinophils, 54.1% for mast cells and 65.3% for epithelial cells. The distribution of neutrophil percentages in both age groups is illustrated in Fig. 1B.

Comparison between age groups–Mucus scores and BALF cell percentages did not differ between Y and O (Fig. 2A & C). The only significant differences in BALF cytology between the groups were a higher total cell count (Fig. 2B) and higher numbers of lymphocytes in Y (42 ± 15 cells/μl) compared to O (28 ± 13 cells/μl). All other absolute numbers of BALF cells did not differ between the two age groups. Variations (CV, Table 1) of all mucus and BALF cytology parameters were very similar in Y and O.

Correlations between age, mucus scores and BALF cytology–Age was negatively correlated with total cell counts ($r = -0.4$) and lymphocyte numbers ($r = -0.6$). No other BALF cytology variables or mucus scores correlated with age. Mucus scores were not significantly correlated with BALF neutrophil percentages and absolute

numbers (Fig. 3A and B). Yet, Fig. 3B shows a trend for the mucus scores to increase with BALF neutrophil numbers.

Discussion

The purpose of this study was to 1) investigate what degrees of airway inflammation and mucus accumulation are present in asymptomatic well-performing sport horses, 2) examine the relationship between airway inflammation and mucus, and 3) compare older to younger individuals.

Subclinical IAD was present in a majority of the healthy well-performing sport horses we studied: Variability and means of airway mucus accumulation and certain BALF cell percentages were considerably higher than normal ranges and means proposed in a recent international workshop on IAD [12]. Because of the absence of eosinophils in most horses and a very high percentage in one horse, this cell type showed by far the highest variation. More than 1% eosinophils has been regarded as an abnormal finding, but increases are often transient and their clinical significance is unclear [184]. Since we did not use a special stain for mast cells, we may have underestimated percentages of this cell type. Nevertheless, mast cell percentages were also higher in our population than observed in healthy racehorses without exercise intolerance [184]. Furthermore, epithelial cell percentages varied considerably between individuals, but no ciliacytophtorea that would indicate subclinical viral infection [185] was detected.

Neutrophils are the hallmark of airway inflammation in RAO [11] and increase with stabling in asymptomatic horses [47, 180], but their role in IAD is unclear [184]. We observed a large variation of this cell type between individuals, independent of their age

(Table 1; Fig. 1A). Both relative (Table 1; Fig. 1B, 2A and 3A and C) and, in particular, absolute numbers (Fig. 3B) were much lower than observed in RAO horses in exacerbation [3]. Compared to recently proposed normal values [12], however, BALF neutrophil percentages in the majority of our subjects would be considered abnormally increased. Our present results suggest that increased airway neutrophils may not only be an acute response to stabling [47, 180], but can be observed in a large proportion of asymptomatic horses housed long-term in a conventional stable environment. We also observed markedly more variation of airway mucus accumulations (Table 1; Fig. 1B) than reported by Dixon *et al.* (1995 [5]) in healthy control horses. This may be due to differences in selection criteria. In the latter study, endoscopic and cytological criteria were used to select healthy controls (McGorum, personal communication), while our present findings are based solely on clinical and historical selection criteria.

Based on our previous findings [179], we had hypothesized that we would find an association between mucus scores and BALF neutrophils (Fig. 1A and B; Table 1), which are known to produce potent secretagogues such as elastase and oxidant radicals [135, 175]. Contrary to our expectations, mucus scores were not significantly correlated with BALF neutrophil percentages (Fig. 3A and B). Fig. 3B suggests, however, that higher mucus scores tended to be associated with BALF neutrophil numbers above 50 cells/μL. We have observed a similar threshold increase in studies with control and RAO-horses [51]. Since most of the horses in this study had less than 100 neutrophils/μL, the relationship may not become statistically apparent. It is possible that the activity of the mucus apparatus is largely independent from lower grade airway inflammation, i.e., <100 neutrophils/μL. The epithelium may react directly to irritants, and there may be important

regional (i.e., large vs. small airways) differences in the intensity of this reaction. This would explain the large variations of mucus accumulation independent from BALF cytological variations we observed in this study as well as the previously reported discrepancy between tracheal aspirate and BALF cytology [186]. Alternatively, BALF cytology, which provides no information on the activity of the observed cells, may simply be too crude a measure of airway inflammation to detect an existing association between subclinical degrees of mucus secretion and inflammatory events in IAD.

Our findings and those of previous survey studies [10, 181] suggest that (subclinical) IAD is present in a majority of stabled horses and may be a normal reaction to the high loads of inhaled irritants [6, 8]. We don't know whether increased levels of airway inflammation and mucus persist in certain individuals. Further studies, which follow horses over time, are needed to address this question. In a clinical setting, however, diagnostic evaluation and treatment decisions are often based on a single examination, similar to that performed in the present study. Even in the absence of clinical signs, BALF neutrophil percentages above 10% and/or more than a few mucus blobs in the trachea (mucus accumulation score > 1) may lead to a diagnosis of IAD. Based on such a simplistic definition, more than half of the examined horses could be diagnosed with IAD. The variation and means of neutrophil, mast cell and eosinophil percentages we observed do not agree with those previously proposed as normal [12]. Indeed, four individuals had neutrophil percentages of more than 15% and three horses had large mucus accumulations.

IAD, even at subclinical levels, is not only an accepted cause of poor performance in racehorses [33], but has also been proposed to affect the ability of sport horses to jump

and perform in dressage [187]. Quite in contrast to that, we found that sport horses performing well over years showed a large range of airway mucus accumulation and BALF inflammatory cell cytology. Consequently, we propose that the significance of different degrees of IAD must be defined separately for different disciplines and levels of equine performance, according to the respective demands placed on the respiratory system and founded on scientific data.

Even though airway inflammation and mucus varied considerably among individuals, we found fewer differences between the two age groups than we had expected. Age is a well-documented factor in the onset of RAO [3], but very little is known about the association of age with milder degrees of airway disease in horses. We hypothesized that years of living in a conventional stable environment, which exposes horses to high dust loads [6, 8], would exacerbate subclinical lower airway disease detectable by endoscopic and BALF examination. However, the present findings lead us to reject this hypothesis. BALF cytology did not show increased inflammation in the older horses (Table 1; Fig. 2A & B), which on average had spent ten more years in the same conventional stable environment than the younger group. Neither did we observe age-related increases of mucus accumulation or apparent viscosity (Table 1; Fig. 2 C), a characteristic finding of long-term irritant exposure in humans [183]. Furthermore, the variations of all BALF and mucus variables were also very similar in the two age groups (CV; Table 1). By definition of our selection criteria, we had eliminated all horses with historical or clinical evidence of lower airway disease from the study. These data therefore suggest that those older horses in their teens, which have remained clinically

healthy despite long-term dust expose, do not suffer increased subclinical airway inflammation and mucus accumulation.

The younger horses, on the other hand, did show higher BALF cell counts and absolute lymphocyte numbers than the older group, a difference reflected in the negative correlation of total cell counts and lymphocyte numbers with age. A lymphocytic type of IAD has been described in young horses and has been compared to the acute form of hypersensitivity pneumonitis [184]. In our view, this may simply reflect an increased "immunological activity", similar, for instance, to the follicular pharyngitis commonly observed in younger horses. Chapman *et al.* (2000 [53]) reported increased "airway inflammation scores", associated with bacterial numbers, in two- and four-year-old compared to older racehorses. However, these "airway inflammation scores" actually represented airway neutrophilia and mucus, variables that showed no age association in our study.

We conclude that older horses, which have remained clinically healthy, did not show increased subclinical airway inflammation or mucus accumulation, while younger horses had moderately higher lymphocyte cell counts. Large interindividual variation of mucus accumulations, and BALF neutrophils, mast cells and eosinophils were observed. Based on previously proposed endoscopic and BALF criteria more than half of the examined healthy and well-performing sport horses had subclinical evidence of IAD. Inclusion of measures of airway obstruction and hyperreactivity may be needed to arrive at a more specific and functionally significant definition of IAD. We further propose that the clinical significance of different degrees of IAD should be judged according to the level of respiratory performance expected from an individual.

Table 4-1: Individual and group signalment, mucus scores and broncho-alveolar lavage cytology.

	Sex	Age	M S	t c c	%lym	%mac	%neu	%eos	%mas	%epi
Y1	G	3	5	110	36.6	35.7	20	0	4.26	3.4
Y2	G	5	4	120	30.5	55.9	7.51	0	5.63	0.47
Y3	F	5	1	170	26.7	63.1	0.97	0.49	3.88	4.85
Y4	G	5	4	70	48	39.1	7.92	0	0.99	3.96
Y5	F	5	3	130	33	32.1	3.83	26.3	2.87	1.91
Y6	G	5	1	110	35.9	55	1.91	0	5.26	1.91
Y7	G	5	2	120	41	41	7.14	0.48	1.9	8.57
Y8	G	6	1	220	39.6	32.3	26.3	0	0.92	0.92
Y9	G	6	0	160	18.9	64.8	8.81	0	7.05	0
Y10	F	6	2	70	36.4	38.7	12	0.46	9.22	3.23
Y11	F	6	3	140	32.6	44.3	10.4	0	8.14	4.52
Y12	G	6	1	90	35	48.5	5.83	0	3.4	7.28
Y13	G	7	1	90	39.4	46.5	6.1	0	4.69	3.29
Y mean		5.4	2.2	123	34.9	45.9	9.1	2.1	4.5	3.4
Y SD		0.9	1.5	31	7.1	11	7.1	7.3	2.6	2.5
Y CV (%)		16.7	68.2	25.2	20.3	23.9	78.0	347.6	57.8	73.5
O1	G	13	3	90	29.2	58.4	27.8	0	3.83	6.22
O2	G	13	1	90	39.5	48.5	1	0	9	2
O3	G	13	2	90	28.4	52.6	14.2	0.95	2.84	0.95
O4	G	13	3	80	31.6	49	13.1	0	1.46	4.85
O5	G	14	2	80	38.1	43.9	5.38	0	7.17	5.38
O6	G	14	3	140	22.3	45.1	22.3	0	6.44	3.86
O7	G	14	1	90	32.9	56.5	4.17	0	3.24	3.24
O8	G	14	1	60	23.4	66.7	1	0	7.46	1.49
O9	G	15	1	100	20.3	58.6	14	0	6.31	0.9
O10	G	15	1	70	42.3	47.8	3.48	0.5	2.49	3.48
O11	G	17	0	80	24.05	51.9	11.2	1.46	3.4	7.77
O12	G	19	2	30	44.5	37.8	0.48	1.91	7.66	7.66
O13	G	19	3	130	51	29.8	0	1.44	10.6	7.21
O mean		14.8	1.8	87	32.9	49.7	9.1	0.5	5.5	4.2
O SD		2.2	1	28	9.5	9.6	8.9	0.7	2.8	2.5
O CV		14.9	55.6	32.2	28.9	19.3	97.8	140	50.9	59.5

Young (Y) and older (O) individuals. Geldings (G) and fillies (F). Mucus scores (MS). Broncho-alveolar lavage total cell counts (tcc; cells/μl), and percentages of lymphocytes (%lym), macrophages (%mac), neutrophils (%neu), eosinophils (%eos), mast cells (%mas) and epithelial cells (%epi). ND: not determined.

Fig. 4-1: Interindividual variability- mucus scores (A) and BALF neutrophil percentages (B) of each horse of both age groups. Ages of the 13 younger and 13 older horses on X-axis.

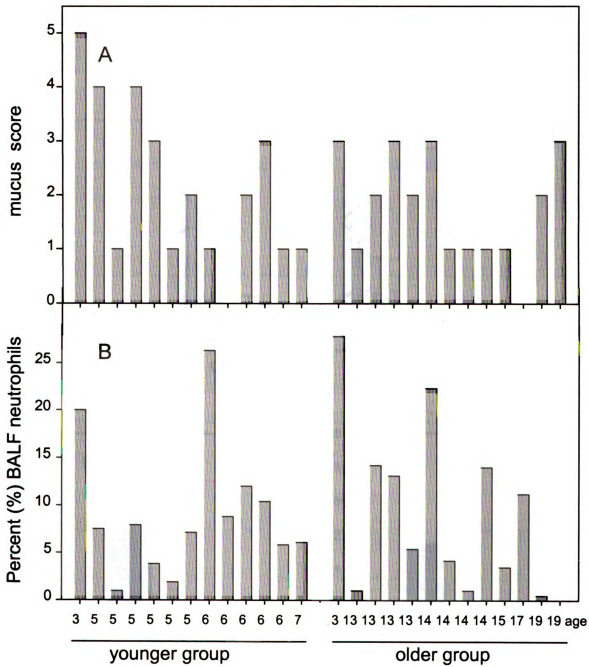


Fig. 4-2: Comparison between age groups (mean±SD)- Proportions (%) of BALF cell populations were not significantly different between the two age groups (A), but younger horses had significantly (*P < 0.05) higher total BALF cell counts (B) than the older group. No difference was observed in mucus scores (C).

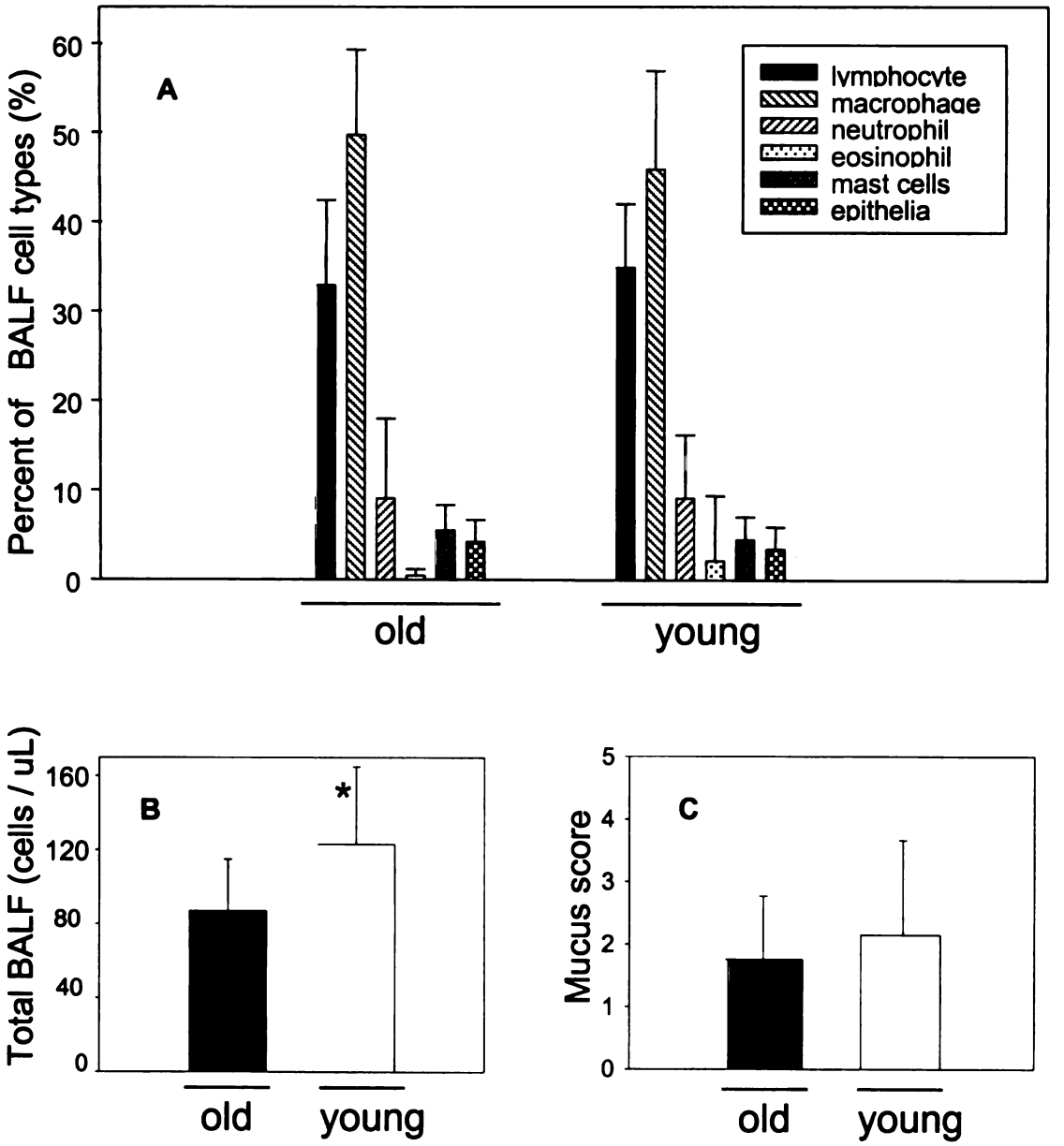
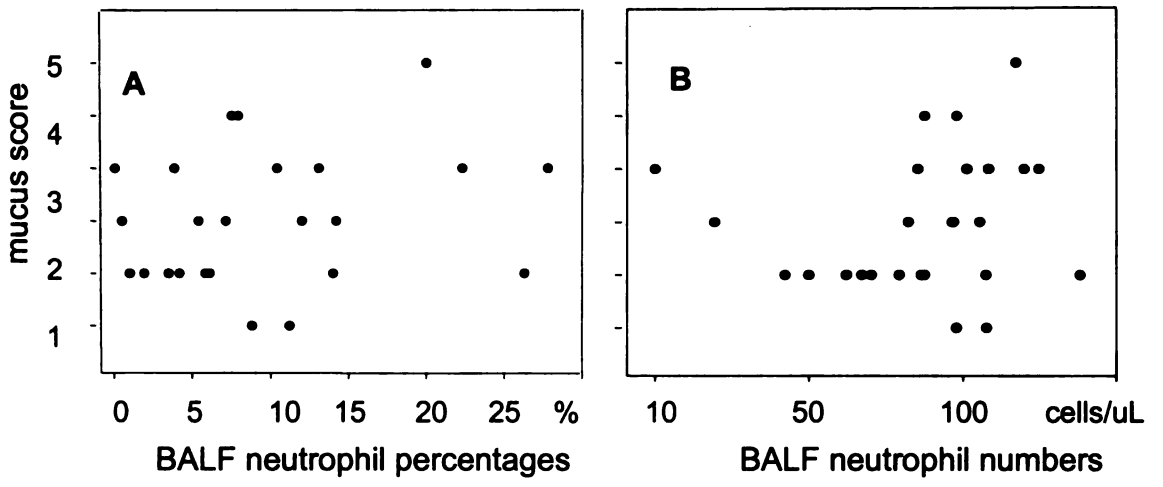


Fig. 4-3: Relationships of mucus quantity with broncho-alveolar lavage (BALF) neutrophil percentages (A) and absolute numbers (B).



CHAPTER 5

MUCUS VISCOELASTICITY AND PREDICTED CLEARABILITY IN RAO-AFFECTED AND CLINICALLY HEALTHY CONTROL HORSES BEFORE AND DURING ENVIRONMENTAL CHALLENGE

Gerber, V., King, M., Schneider, D.A., Robinson, N.E. Tracheobronchial mucus viscoelasticity during environmental challenge in horses with recurrent airway obstruction. (2000). *Eq Vet Journal.*

The previous studies (chapter 1-3) I showed that airway mucus accumulation is a function of disease status interacting with environment, but not of age. Based on these results, estimated volumes of mucus transported along the trachea in clinically healthy horses are similar to healthy humans and dogs, but are increased 2-fold in RAO-affected horses in remission and more than 4-fold during exacerbation.

In order to test the hypothesis that these increases in airway mucus accumulation in remission as well as during exacerbation may be caused by unfavorable alterations of mucus rheological properties, I compared mucus viscoelasticity (and predicted clearability) in RAO-affected and clinically healthy control horses before (when they were at pasture) and after (6, 24 and 48 hours) stabling on straw bedding and hay feeding.

Abstract

The goal of this study was to compare the rheological properties of mucus from horses with recurrent airway obstruction (RAO) to that from healthy controls during environmental challenge by stabling in stalls with straw as bedding and hay as feed. We determined and report viscoelasticity (log G* dyn/cm^2, at 10 radian/s) and calculated and report mucociliary clearability index (MCI) and cough clearability index (CCI), which are derivative parameters of G* and the ratio of viscosity and elasticity measured at 1 and 100 radian/s, respectively. We also investigated the solids content of mucus, and cytology of bronchoalveolar lavage fluid (BALF). Samples were obtained before (0 hours) and 6, 24 and 48 hours after environmental challenge. The central findings were rheological changes in airway mucus, which occurred over time in RAO-affected animals, but not in controls. Mucus rheology was similar in both groups at 0 and 6 hours. In RAO-affected horses, mucus viscoelasticity, as measured by log G*, increased from 2.49 ± 0.18 dyn/cm^2 (mean ± s.e.m.) at 0 hours to 3.05 ± 0.13 dyn/cm^2 at 24 hours after environmental challenge, and was accompanied by significant decreases in MCI and CCI. Percent solids of mucus did not significantly differ between the two groups nor over time. Rheological values did not correlate with BALF cytology. We conclude, that viscoelastic properties of tracheal mucus samples from RAO-horses in remission do not differ from those of normal horses. However, environmental challenge causes clinical signs of acute airway obstruction and a concurrent increase in mucus viscoelasticity only in RAO-horses. Therefore, we infer that unfavorable changes in mucus rheology may contribute to stasis and accumulation of mucus in RAO-horses in exacerbation, but not in clinical remission.

Introduction

Excess mucus accumulation in the airways is a hallmark of recurrent airway obstruction (RAO) [11]. We have shown that mucus accumulation scores are higher in RAO than in controls, and increase further in RAO-affected animals when they are stabled and exposed to hay [182]. These accumulations may be due to increased secretion and/or decreased clearance. In turn, alterations in the ciliary apparatus or in the physical properties of mucus ultimately result in changes of mucociliary clearance [69, 188]. The effectiveness of mucociliary clearance in horses with RAO, however, is controversial. Some investigators demonstrated that the rate of mucociliary clearance is decreased in RAO-affected animals [58, 64], while others found no difference compared to healthy horses [65]. Studies on excised pieces of trachea from healthy horses have shown that variations of ciliary beat frequency at physiological temperatures have only a minor effect on mucociliary clearance rate. In contrast, the properties of mucus are very important [188].

We use the term "mucus" to describe the entity of gel-like secretions that are present in the airways and available for sampling and rheological analysis. The composition of airway mucus is heterogeneous, composed of water, mucin glycoproteins, lipids, as well as cells with their degradation products and serum-derived proteins. This heterogeneity imparts both liquid-like (viscous) and solid-like (elastic) properties to this complex viscoelastic gel. Fig. 1 illustrates the relationships between viscoelasticity (G*), the vector sum of viscosity (G") + elasticity (G'), and the ratio of viscosity / elasticity (tan δ). Alterations in these viscoelastic properties of mucus result in changes in its clearability by ciliary and cough mechanisms [69]. A critical factor affecting the

viscoelasticity of mucus is hydration, i.e., its relative solids and water content [189, 190]. Another important factor is the presence of inflammatory cells. In particular, high molecular weight DNA [171, 191] and filamentous actin [192] released by degrading neutrophils increase mucus viscoelasticity in cystic fibrosis and possibly also in chronic bronchitis and asthma. In these human airway diseases rheological alterations of mucus are thought to be associated with the severity of disease (reviewed by Kim 1997 [39]).

In RAO-affected horses, neutrophils invade the lung within 7 hours of environmental challenge [14] and accumulate in the airways in large numbers [15]. Airway obstruction develops concurrently. Although stasis and accumulation of mucus in the airways is thought to play an important role in the pathogenesis of RAO [3], there is little information on the rheology of equine respiratory mucus [21, 172].

The purpose of this study was to investigate the rheological properties of equine respiratory mucus. We hypothesize that in RAO-affected horses in exacerbation, airway inflammation is associated with compromised clearability of mucus. Therefore, we compared the rheological properties of mucus from horses with RAO and healthy controls, before and after environmental challenge by stabling in stalls with straw as bedding and hay as feed. We measured viscoelasticity of tracheal mucus samples, computed the derivative parameters of predicted mucociliary and cough clearability and determined the solids content. Furthermore, we investigated the association of rheological properties with the solids content of mucus and with the cytology of bronchoalveolar lavage fluid (BALF).

Materials and methods

Animals and study design–This study was approved by the All-University Committee for Animal Use and Care of Michigan State University. Seven horses (3 mares and 4 geldings), 9 to 26 years old (mean ± s.e.m., 17.3 ± 2.4 years), with a previous diagnosis of RAO, served as the principal group. Seven horses (5 mares and 2 geldings), aged between 7 and 26 years (mean ± s.e.m., 13.4 ± 2.8), and with no evidence of respiratory tract disease, served as the control group. Both groups of horses were drawn from closed research herds of the Department of Large Animal Clinical Sciences, Michigan State University, that have been maintained for several years and develop (principal group) or do not develop (control group) signs of obstructive pulmonary disease when stabled and fed hay. All horses were vaccinated against equine influenza, rhinopneumonitis, Eastern and Western equine encephalitis.

Clinical signs of airway obstruction were numerically scored as defined below. Horses were kept at pasture and their diet was supplemented with pellets until all animals had no clinical signs of airway obstruction. Clinical assessment and samples of mucus and BALF were obtained at baseline (0 hours) and 6, 24 and 48 hours after environmental challenge by stabling in stalls with straw bedding and feeding hay.

Clinical score (CS)–One observer (Gerber) using a numerical scoring system clinically assessed severity of airway obstruction. Each horse was examined at rest, before sample collection. Nostril flare was scored on a scale of 0 (least severe) to 2 (most severe) and abdominal compression on a scale of 0 (least severe) to 3 (most severe). For nostril flare: 0 = no or little movement on inspiration; 1 = flare during inspiration, returning to normal as inspiration ends; 2 = flare during inspiration and exhalation. For

abdominal lift: 0 = no or little movement in the ventral region of the flank; 1 = slight abdominal flattening in the ventral region of the flank; 2 = obvious abdominal flattening and "heave line" extending no more than half way between the cubital joint and the tuber coxae; 3 = obvious abdominal lift and "heave line" extending beyond halfway between the cubital joint and the tuber coxae. CS was calculated by adding the scores from nostril flare and abdominal lift. In order to enter the study, all animals had to have CS $\leq$ 1 at 0 hours; i.e., RAO horses were in clinical remission at the beginning of the study. By definition, CS had to remain $\leq$ 1 at all time points (0, 6, 24 and 48 hours) for all animals of the control group. Horses belonging to the principal group had to develop CS $\geq$ 2 at some time point after stabling (6, 24, 48 hours).

Collection and analysis of mucus samples–Mucus was obtained in the trachea by means of a cytology brush passed via an endoscope, similar to the technique used in humans [73]. The sheath of a soft-haired bronchial brush (Olympus[1] BC-23Q) was removed, the wire of the brush glued into a teflon catheter and thus modified for use with a 3 meter-endoscope (Olympus[1] GIF 300, custom made). The working channel of the endoscope was thoroughly flushed and then completely dried with compressed air each time before the brush was introduced. As previous results had shown that equine mucus sampled in the dorsal vs. the ventral part of the trachea differs in its average rheological properties [179], we aimed to reduce the variability by sampling mucus only in the ventral half of the trachea. After sampling, the brush was retracted 2-3 cm into the working channel and the endoscope was withdrawn from the animal. The brush was again protruded and mucus removed from the brush to be immediately stored under light

mineral oil at -80°C. For the subsequent rheological measurements the samples were rapidly thawed to room temperature.

The magnetic microrheometer was used to measure the bulk viscosity and elasticity [168]. A 100 μm steel ball was carefully positioned in a 2 -10μL sample of mucus and oscillated by means of an electromagnetic field gradient. The motion of this sphere was tracked with the aid of a photocell. Plots of ball displacement versus magnetic force were used to determine the viscoelasticity (G*; Fig. 1) and tangent δ (tan δ: Fig. 1). G* (dyn/cm^2) and tan δ were measured at frequencies of 1, 10 and 100 radian/s. We report viscoelasticity as log^{10}, (log G*) at 10 radian/s. For purposes of comparison, G* of water is 0.01 (log -2) dyn/cm^2 and that of resin is 100 (log 2) to 1000 (log 3) dyn/cm^2. For a perfect solid, tan δ is zero; for a perfect liquid, tan δ is infinite. From these measured rheological properties the derived parameters mucociliary clearability index (MCI) and cough clearability index (CCI) were computed based on observations of clearance in model studies [69]. MCI, indicating clearability by normal ciliary function, was calculated based on log G* and tan δ measured at 1 radian/s, and CCI was calculated based on log G* and tan δ at 100 radian/s. Both indices relate negatively with log G*; MCI also relates negatively with tan δ, but CCI relates positively with it. The respective formulae are as follows:

$$MCI = 1.62 - (0.22 * \log G^*_1) - (0.77 * \tan \delta_1) \quad (1)$$

$$CCI = 3.44 - (1.07 * \log G^*_{100}) + (0.89 * \tan \delta_{100}) \quad (2)$$

After rheological analysis, the solids content of mucus was determined. Samples were rinsed in petroleum ether to remove adherent paraffin oil, weighed on glass slides and evaporated to dryness in a microwave oven (30 min at 750 W). The ratio of dry to

wet weight was calculated and expressed as % solids content. Samples less than 2 mg were excluded from the % solids analysis.

Bronchoalveolar lavage fluid (BALF) sampling and analysis–BALF was collected by means of a 3-meter endoscope (Olympus[1] GIF 300, custom made) that was passed via the nares and wedged in a peripheral bronchus. Six 50-mL aliquots of phosphate buffered saline (PBS) were infused into the tube and recovered by suction. The lavaged fluids were pooled and the total recovered volume determined. Total cell counts in BALF were performed manually using a hemacytometer. Cell smears were made with a cytocentrifuge and stained with Diff-Quick. Differential cell counts of lymphocytes, macrophages, neutrophils, eosinophils, mast cells and epithelial cells were performed by counting 100 cells per sample.

Statistical analysis–All statistical analyses were performed using a statistical software program (SAS[2] for Windows95, version 6.12). Four dependent measures on tracheal mucus were analyzed by multivariate ANOVA for repeated measures using the general linear model. Mucus viscoelasticity (log G* measured at 10 Hz), the two rheological derivatives (MCI and CCI) and the % solids were the dependent measures of interest. Independent factors in the model included two levels (principal and control horses) of the between subjects factor, group, and four levels (0, 6, 24, and 48 hours) of the repeated measure, time. Subanalyses were performed as predetermined specific linear contrasts or as simple effects and interactions when main or interaction effects were significant. BALF total and differential cell counts (total cell count X percentage of each cell type) were expressed as the base 10 logarithmic transformation to satisfy the model assumptions of equal variance and normal distribution. A constant was first added to

these data in order to make all data non-zero for logarithmic transformation. The transformed BALF differential cell counts were then similarly analyzed. Pearson's partial correlation analysis was used to probe the relationships of three rheological measures (log G*, MCI, and CCI) and the % total solids of tracheal mucus with the differential cell counts from BALF. Partial variables included the two levels of group and four levels of time. For all analyses, significant differences were declared if $P < 0.05$. All data is herein expressed as the mean (s.e.m.).

Results

Clinical score (CS)–The fourteen horses entering the study in the principal (n=7) or control group (n=7) qualified according to the respective criteria for CS as defined above. When entering the study, all animals had CS $\leq$ 1 (0 hours). All individuals of the principal group developed CS $\geq$ 2 at some time point after stabling; at 0 hours CS was 0.57 ± 0.20, at 6 hours 1.14 ± 0.26, at 24 hours 2.86 ± 0.55 and at 48 hours 2.57 ± 0.43. CS remained $\leq$ 1 in all animals of the control group at all time points (0.14 ± 0.14, 0.0 ± 0.0, 0.14 ± 0.14 and 0.0 ± 0.0 at 0, 6, 24 and 48 hours, respectively).

Biophysical properties of mucus secretions–The rheological measures, log G* and tan δ at 10 radian/s, for each horse are presented in Table 1. All parameters of mucus physical properties are summarized in Table 2. Figs. 2A, 2B and 2C illustrate log G* at 10 radian/s, MCI and CCI, respectively.

A complete set of measurements of log G* and tan δ at 10 radian/s from 56 samples was obtained. In the principal group baseline values (0 hours) of log G* averaged 2.49 ± 0.18 dyn/cm^2 (Table 2; Fig. 2A). At 6 hours values had decreased to 2.32

± 0.13 dyn/cm^2. With the exception of horse 5, the highest values of each individual were observed at 24 (Table 1: horses 1, 2 and 3) and 48 hours (Table 1: horses 4, 6 and 7). This resulted in an increase of the average to 3.05 ± 0.13 and 2.83 ± 0.10 dyn/cm^2, respectively (Table 2; Fig. 2A). In the control group (Table 1: horses 8-14), with the exception of horse 11, values of log G* at 10 radian/s were below 2.9 dyn/cm^2 and no values of 3 dyn/cm^2 or above were observed. On average log G* (all control group values pooled 2.40 ± 0.06) was similar to the principal group baseline values and remained so during stabling (Table 2; Fig. 2A). There was a significant difference between principal and control log G* values (main group effect, P = 0.0187) which depended on time (interaction effect of group and time, P = 0.0161). Subanalysis determined that log G* values were not different between groups at baseline (P = 0.759) and that log G* did not change over time in control horses (P = 0.746). In contrast, log G* was increased in principal horses at 24 hours when compared to baseline (P = 0.041) and at 24 and 48 hours when compared to time-matched control values (P = 0.002 and 0.003, respectively).

Values of tan δ were similar between the groups and over time (Table 1 and 2). None of the main or interaction effects were significant. The average of all tan δ values at 10 radian/s, principal and control group pooled, was 0.37 ± 0.01.

A complete set of measurements of log G* and tan δ at 1 radian/sec from 56 samples was available to compute MCI. (Table 2 and Fig. 2B). There was a significant difference between principal and control MCI values (main group effect, P = 0.031). However, a significant main or interaction effect of time was not detected. Subanalysis determined that MCI values were not different between groups at baseline (P = 0.935)

and that MCI values did not change over time in control horses (P = 0.901). The difference between principal and control MCI values was significant at 24 hours (P = 0.011) and the difference between principal and control MCI values at 24 hours was significantly greater than at baseline (P = 0.043). A significant simple effect of time on the principal MCI values was not detected (P = 0.209).

CCI was computed for 49 samples, 7 measurements of log G* were not possible at 100 radian/s due to high viscoelasticity of the samples. Only 6 control and 3 principal horses were retained in the analysis due to these missing values. There was a significant difference between principal and control CCI values (main group effect, P = 0.012). However, a significant main or interaction effect of time was not detected. Subanalysis determined that CCI values were not different between groups at baseline (P = 0.616) and that CCI values did not change over time in control horses (P = 0.478, n = 6). The difference between principal and control CCI values was significant at 24 hours (P = 0.028) and the difference between principal and control CCI values at 24 hours was significantly greater than at baseline (P = 0.043, n = 5). At 48 hours, the P-value for the difference between principal and control CCI values was 0.061. A significant simple effect of time on the principal CCI values was not detected (P = 0.316).

The % solids content was measured in 54 samples (Table 2). Two samples of less than 2 mg wet weight were discarded, and due to missing values one control horse was excluded from this analysis. There were no significant main or interaction effects. The average of all pooled samples (n=54) was 9.8 ± 0.4 % solids. No significant correlations were observed between % solids and log G*, MCI and CCI.

BALF cytology–Fifty-three BALF samples were available for cytological evaluation. Summary data are presented in Table 3. Total cell count increased more than three-fold (0 vs. 48 hours) and % neutrophils more than four-fold (0 hours vs. 48 hours) in the principal group during environmental challenge. The following statistical results were obtained on $\log^{10}$ transformed BALF cell counts. There was a significant effect of group ($P = 0.01$) and time ($P = 0.014$) on the total cell count. The P-value of the interaction effect of group and time was 0.064. Subanalysis determined that the total cell counts were not different between groups at baseline ($P = 0.167$) and that total cell counts did not change over time in control horses ($P = 0.873$). In contrast, the total cell count increased in principal horses at 6 hours when compared to baseline (0.003), and at 6 and 24 hours when compared to time matched control values ($P = 0.032$ and 0.022, respectively). There was a significant effect of group ($P = 0.001$) on the neutrophil count, which depended on time (interaction effect of group and time, $P = 0.001$). Subanalysis determined that the neutrophil counts were not different between groups at baseline ($P = 0.258$) and that neutrophil counts did not change over time in control horses ($P = 0.13$). In contrast, the neutrophil count was increased in principal horses at 6, 24, and 48 hours when compared to baseline ($P = 0.002$, $P = 0.001$, and $P < 0.001$, respectively). For lymphocyte, eosinophil, mast, macrophage, and epithelial cell counts, there were no main or interaction effects. There were no significant partial correlations of BALF cytology with either log G*, MCI, or CCI.

Discussion

To our knowledge, no studies have directly compared mucus from RAO-affected horses and healthy controls. While other rheological methods require relatively large quantities of mucus, microrheometry allows for investigation of mucus rheology even in healthy subjects, whose airways contain only small amounts of secretions [73].

At the start of the protocol and six hours after the horses had been stabled and fed hay, viscoelasticity and clearability of mucus did not differ significantly between RAO-affected horses and controls. The values were in the range of those previously observed in healthy human subjects [73] and in healthy dogs, but lower than in smaller species, i.e., ferrets, rabbits and rats [193]. Since mucus rheology was not different from controls in the RAO-affected animals before stabling, accumulations of secretions observed when RAO-horses are in remission are likely be due to other than rheological factors (e.g., hypersecretion). However, twenty-four hours after the environmental challenge, the viscoelasticity of mucus had significantly increased, log G* averaging 3.0 ± 0.3 dyn/cm^2 (Table 2; Fig. 2A). This change of log 0.5 represents a 3-fold increase of viscoelasticity on a linear scale. Furthermore, log G* values of 3 and more are in the range found in human patients suffering from severe airway obstruction caused by cystic fibrosis [194].

The ratio of viscosity / elasticity did not differ between controls and RAO-horses and was not significantly affected by stabling and hay challenge. This means that the observed increase of viscoelasticity in the principal group was due to proportional changes of both viscosity and elasticity. Values for tan δ were similar to those reported in healthy humans [73] and in other species [193].

As a result of the changes in viscoelasticity, both predicted mucociliary and cough clearability in RAO-affected horses were significantly affected by the environmental challenge, MCI reflecting the changes in log G* more closely (table 2; Fig. 2A, B and C). These results suggest that the changes in viscoelasticity, which occur at the onset of clinical signs, affect both ciliary and cough clearability of mucus and, may therefore contribute to accumulation and stasis of mucus in the airways of RAO-affected horses in acute exacerbation. It is important to note that, while our findings suggest unfavorable changes in mucus clearability based on calculated MCI and CCI, factors other than viscoelasticity such as the spinnability or the adhesivity of mucus for instance, which we have not investigated in this study, may significantly influence clearability. Also, the changes in predicted MCI and MCI were most pronounced at 24 hours of environmental challenge. After another day in the stable, both viscoelasticity and mucociliary clearability values showed a trend of returning towards baseline. The very high values of viscoelasticity we observed may be a transient phenomenon of acute exacerbation occurring after stabling highly susceptible horses.

We have previously investigated mucus rheology in horses with only mild to moderate clinical signs of airway obstruction (i.e., chronic accumulation of mucus in the airways, coughing, but no dyspnea) [195]. The results of this investigation contrast with the findings of the present study. These horses with mild to moderate clinical signs showed remarkably lower average viscoelasticity (mean ± SD: 1.96 ± 46) and better clearability (MCI mean ± SD: 0.99 ± 0.11) than the principal and even the control horses in the present study. Our principal group, of course, was composed of severely RAO-affected animals, which by definition showed marked clinical signs when stabled and fed

hay. Also, the mean age of both the principal and control horses of the present study was 7 and 3 years higher, respectively, than that of the mild to moderately affected horses. Interestingly, in heavy smokers an age (pack-years)-dependent decrease followed by an increase in viscoelasticity has been observed [183]. Age may play a role in equine mucus rheology as well. In the present study, however, age was not a statistically significant factor either between or within the principal and the control group (results not shown).

Since the mucus samples were harvested, stored and analyzed by the same methods, the disparity in the rheology of the mucus between the present and the previous findings must result from either the above mentioned differences in severity of disease and age of the animals or in the differing design of the two studies. While the horses in the present study were exposed to hay after having been at pasture for an extended period, the mild to moderately affected animals in the previous study were stabled and fed hay for at least three weeks prior to sampling mucus.

Based only on the physical appearance of tracheobronchial secretions, Schatzmann et al. (1972 [45]) proposed that mucus viscosity is proportional to the severity of clinical signs of airway obstruction in RAO-horses. When these authors measured viscosity of mucus in 5 RAO-affected horses in a subsequent study they found it to be lower than in human patients with severe chronic bronchitis [21]. However, prior to the trial, hay had been eliminated from the diet of these animals [21]. A recent report indicated significantly higher values of mucus viscosity in horses with RAO than in horses affected with inflammatory airway disease [172]. The authors proposed that the difference was due to the presence of high molecular weight DNA in the mucus of the RAO-affected animals. Unfortunately, due to differing methods and limited published

information no direct comparison is possible with these two reports [21, 172]. The available data on equine respiratory mucus rheology suggest, however, that alterations in mucus rheology in horses may be a function of the intensity and duration of exposure to irritants and allergens as well as the severity and duration of airway disease in the individual. This could explain contradictory findings reported in studies of mucociliary clearance in RAO-affected horses [58, 64, 65], since clearance may be either normal or compromised depending on individual and environmental factors. Further studies are needed to determine the respective influences and interaction of intensity and duration of exposure as well as the severity and duration of disease in the individual on changes in mucus rheology.

The present study was not designed specifically to identify mechanisms responsible for changes in mucus rheology. Hydration of mucus (i.e., % solids) was not significantly affected by stabling and did not seem to play a role in the rheological changes we observed. BALF cytology before and during environmental challenge showed the typical differences between RAO-affected horses and controls [3]. However, BALF and tracheal aspirate cytology lack a strong correlation [186]. This, and the stringency of the statistical test we employed, may explain why we did not observe any significant correlations between parameters of BALF cytology and tracheal mucus rheology. Therefore, our results do not support any predictive or diagnostic value of BALF cytology for rheological changes induced by the environment; but neither do they rule out that inflammatory cells, or more specifically their degradation products, may have an effect on mucus rheology. Interestingly, preliminary mucolytic data based on a limited number of samples from this study suggest that extracellular filamentous actin is

present in mucus from RAO-affected animals and, possibly in combination with DNA fibers, increases its viscoelasticity (Gerber, unpublished results).

The central finding of this study was that environmental challenge by stabling and hay diet unfavorably alters mucus rheology in RAO-affected horses, but not in controls. We conclude, that viscoelastic properties of tracheal mucus samples from RAO-horses in remission do not differ from those of normal horses. During the onset of clinical signs of airway obstruction, however, mucus viscoelasticity in RAO-horses increases to values observed in severe obstructive lung disease of humans, reducing both ciliary and cough clearability. Therefore, unfavorable changes of mucus rheology may contribute to stasis and accumulation of mucus in the airways of RAO-affected horses in exacerbation, but not in remission.

Table 5-1: Biophysical properties of individual mucus samples: Viscoelasticity (log G*; dyn/cm^2) and tangent δ, measured before (0 hours) and during (6, 24, 48 hours) stabling in each RAO horse (1-7) and each control (8-14).

	Viscoelasticity (log G* at 10 radian/s; dyn/cm^2)				tangent δ (at 10 radian/s)			
hours after stabling	0	6	24	48	0	6	24	48
				Principal group				
horse 1	2.39	2.47	3.32	2.30	0.23	0.36	0.32	0.24
horse 2	1.89	2.57	3.07	2.91	0.29	0.31	0.47	0.33
horse 3	2.58	2.65	3.61	2.86	0.34	0.31	0.32	0.30
horse 4	2.22	1.87	2.8	2.93	0.29	0.41	0.51	0.54
horse 5	3.27	2.66	2.81	2.71	0.33	0.54	0.4	0.32
horse 6	2.95	2.13	3.16	3.17	0.36	0.29	0.41	0.19
horse 7	2.15	1.91	2.57	2.92	0.36	0.48	0.37	0.43
				Control group				
horse 8	2.49	2.57	2.11	2.51	0.32	0.46	0.36	0.35
horse 9	2.66	2.70	2.31	2.15	0.39	0.28	0.34	0.29
horse 10	2.66	2.38	2.24	2.52	0.35	0.37	0.35	0.39
horse11	2.34	2.99	2.90	2.54	0.35	0.36	0.38	0.32
horse 12	1.74	2.52	2.50	2.48	0.30	0.50	0.43	0.40
horse 13	2.89	1.68	2.50	2.00	0.39	0.56	0.35	0.68
horse 14	2.16	2.53	2.08	2.16	0.36	0.42	0.37	0.35

Fig. 5-1: Vector diagram illustrating the viscoelasticity of mucus (G*), the viscous (G'') and elastic (G') components and their ratio defined by tangent δ.

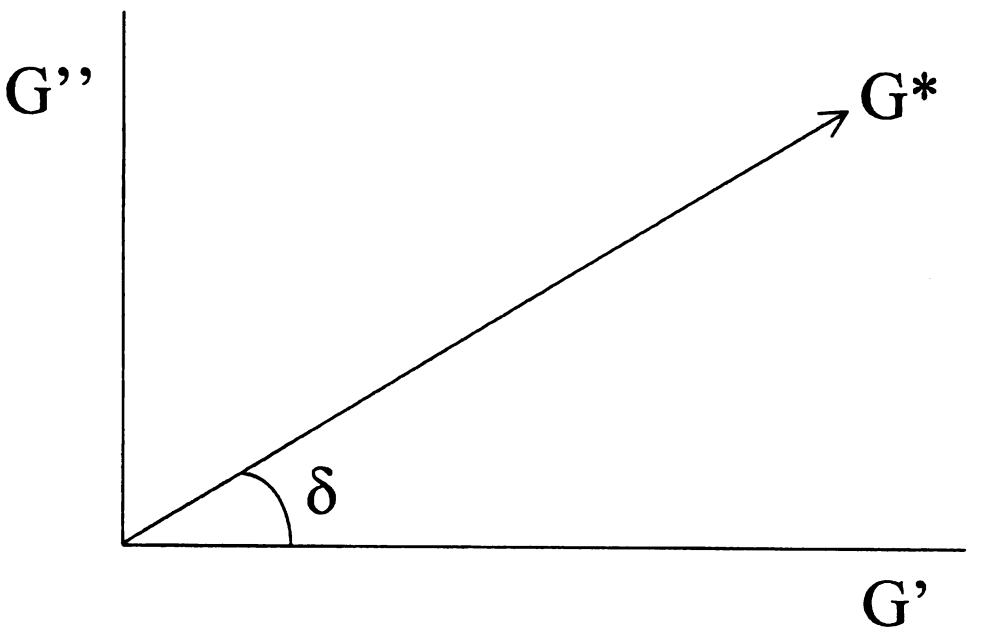
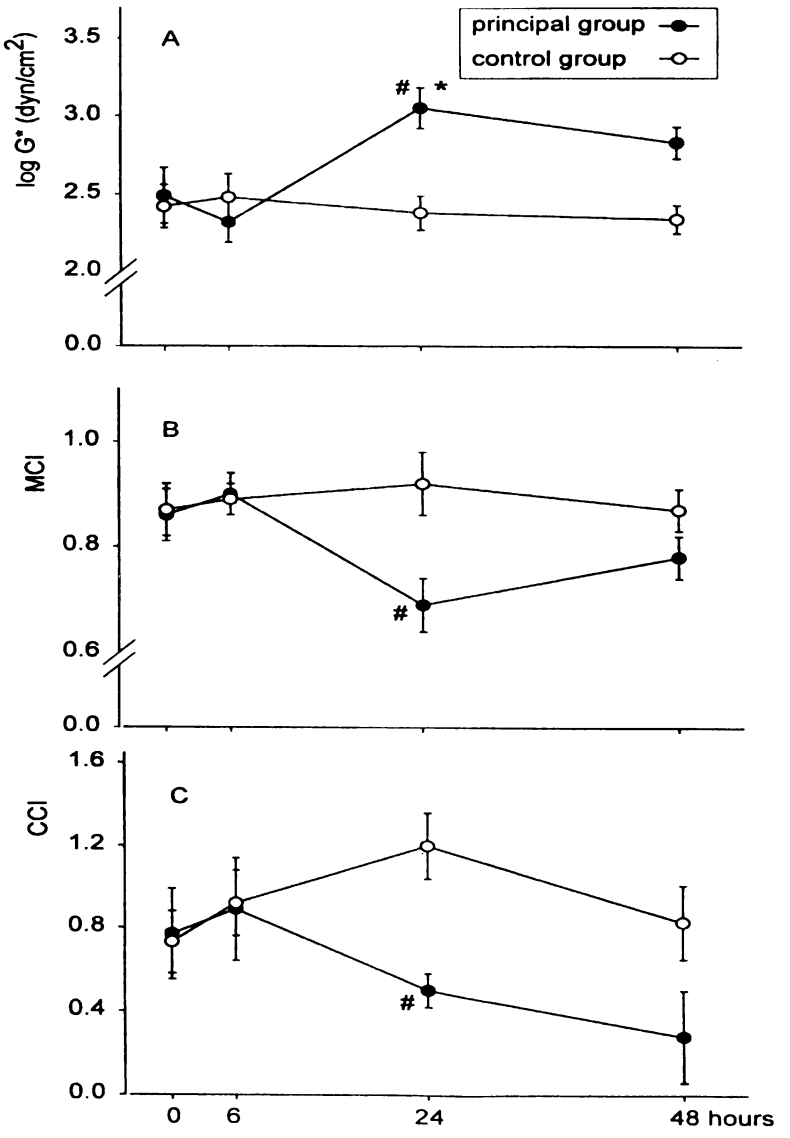


Fig. 5-2: A) viscoelasticity at 10 radian/s on a logarithmic scale (log G*; dyn/cm^2), B) mucociliary clearability index (MCI) and C) cough clearability index (CCI) of mucus samples in RAO-affected (principal group) and control horses (control group) before (0) and after environmental challenge (6, 24 and 48 hours). # Indicate significant differences between principal and control group at a time point. * Indicates a significant difference between the time point and baseline in the same group.



CHAPTER 6

PRELIMINARY STUDY ON THE MUCOLYTIC EFFECTS OF DNase AND GELSOLIN ON EQUINE MUCUS RHEOLOGY–F-ACTIN MAY CONTRINUTE TO INCREASED VISCOELASTICITY DURING RAO EXACERBATION

In the previous study (chapter 5) I showed that airway mucus viscoelasticity increased 3-fold in RAO-affected horses during exacerbation. This increase coincides with a dramatic influx of neutrophils in the airways of RAO-affected horses. Neutrophils in the airways degenerate and release breakdown products, specifically DNA and/or filamentous (F)-actin that can unfavorably alter mucus rheological properties.

In order to test the hypothesis that these breakdown products cause unfavorable rheological changes, i.e., the observed increased mucus viscoelasticity during RAO exacerbation, I investigated the effects of DNase, which degrades DNA, and gelsolin, which degrades F-actin, on airway mucus from RAO-affected in exacerbation compared with control mucus samples in a preliminary mucolytic study.

Abstract

We demonstrated that the viscoelasticity of mucus in recurrent airway obstruction (RAO)-affected horses in remission is similar to that of healthy controls. In acute exacerbation after stabling and hay exposure, however, mucus viscoelasticity increases 3-fold in RAO-affected horses, but remains normal in controls. This increase in viscoelasticity coincides with a large neutrophil influx in the airways. High molecular weight DNA fibers and filamentous (F) -actin from degrading neutrophils increase mucus viscoelasticity in human cystic fibrosis (CF). The importance of DNA fibers in RAO mucus is unclear, however, and F-actin has not been investigated. The aim of this preliminary study was to investigate the role of these neutrophil degradation products in the unfavorable rheological changes observed during RAO exacerbation. We assessed the mucolytic effects of gelsolin, a capping protein that severs F-actin filaments, and rhDNase, which cleaves high molecular weight DNA molecules. Concentrations of gelsolin and rhDNase previously shown effective on CF sputum were tested on mucus from RAO-affected horses in exacerbation compared with control mucus from RAO-affected horses in remission and from clinically healthy horses. Negative and positive control treatments (sham, saline and nacystelyn; DNase and gelsolin on CF mucus) confirmed the validity of mucolytic experiments. RhDNase treatments did not alter viscoelasticity in either RAO or control mucus. Gelsolin had a significant mucolytic effect on mucus of RAO horses in exacerbation, effectively reducing viscoelasticity almost 3-fold to normal levels. Unlike the control treatment with nacystelyn, gelsolin had no effect on control mucus. This indicates that F-actin plays a role in unfavorable rheological changes observed in mucus of RAO-

affected horses during exacerbation. In contrast, DNA fibers either do not play a similarly important rheological role in RAO mucus as in CF mucus, or, alternatively, they may be protected to a larger degree from mucolytic action. F-actin fibers have been shown to form an entangled network with DNA fibers, not only causing increases in viscoelasticity, but also protecting DNA fibers from mucolytic action by rhDNase. Further studies are needed to elucidate the role of DNA and F-actin fiber interactions in RAO mucus. In conclusion, this preliminary study showed that RAO mucus is similarly susceptible to the action of gelsolin as CF sputum, while higher concentrations of rhDNase may be necessary to achieve a mucolytic effect.

Introduction

Equine recurrent airway obstruction (RAO) is characterized by bronchospasm and intense neutrophilic inflammation with inflammatory cells and cellular debris, serum proteins and mucus glycoproteins accumulate in the airways [2, 3]. All these components of mucous secretions can influence the viscoelasticity of mucus, which in turn is associated with mucus clearability by mucociliary and cough mechanisms [69, 196-198]. We demonstrated that the viscoelasticity of mucus in RAO affected horses in remission is similar to that of healthy controls. In acute exacerbation, however, mucus viscoelasticity in RAO increases 3-fold [169] to levels otherwise only observed in human cystic fibrosis (CF) [194]. This increase in viscoelasticity coincides with an increase of endoscopically observed mucus accumulation [182] and with neutrophil influx [169] in the airways, but is not associated with changes in the hydration of the mucus [169].

High molecular weight DNA [171] and filamentous (F) -actin [192] released by degrading neutrophils increase mucus viscoelasticity in CF and possibly also in chronic bronchitis and asthma. DNA and F-actin can form a network within the existing mucin network. The relative importance of this DNA/f-actin network in human airway diseases is unclear, but sputum stained to differentiate mucin from DNA and actin showed that there are large amounts of mucin and little DNA in chronic bronchitis sputum, but relatively less mucin and much more DNA and actin in CF sputum (M. King, personal communication).

In the horse, Schatzmann et al (1973 [21]) found that free DNA was not present in excess in mucus of RAO-affected horses compared with sputum of human chronic bronchitis patients. In contrast, Pietra et al. (2000 [172]) have shown a mucolytic effect of human(h) recombinant(r) DNase on mucus of RAO-affected horses. Thus, the importance of DNA fibers is unclear and F-actin has not been investigated in equine airway mucus.

The aim of this preliminary study was therefore to investigate the role of these neutrophil degradation products in the unfavorable rheological changes observed during RAO exacerbation. We assessed the mucolytic effects of gelsolin, a capping protein that severs F-actin filaments, and rhDNase, which cleaves high molecular weight DNA molecules. Concentrations of gelsolin and rhDNase effective on CF sputum [192, 194] were tested on mucus from RAO-affected horses in exacerbation.

Materials and methods

Horses, mucus sampling and rheological measurements–Seven RAO-affected (9 to 26 years old) and 7 healthy control (7 to 26 years old) horses were kept

at pasture and their diet was supplemented with pellets until all animals had no clinical signs of airway obstruction. Mucus samples were obtained at baseline (0 hours) and 6, 24 and 48 hours after environmental challenge by stabling in stalls with straw bedding and feeding hay. Mucus was sampled from the ventral part of the trachea by means of a cytology brush passed via an endoscope [73] and immediately stored under light mineral oil at -80°C as described [169]. The magnetic microrheometer technique was used to measure viscoelasticity (the vector sum of viscosity and elasticity: G* (dyn/cm^2)) and tan (the ratio of viscosity and elasticity) at frequencies of 1, 10 and 100 radian/s as described [168].

Mucolytic treatment–Gelsolin (Biogen, Inc. Cambridge, MA) and rhDNase (Pulmozyme®, Genentech, Inc. San Francisco, CA) were applied in saline at 10% of sample weight. Gelsolin and rhDNase concentrations were 200nM, as previously shown effective in CF sputum [199]. Nacystelyn (SMB pharmaceuticals, Belgium), which acts by breaking up disulfide bonds between mucin molecules, was used as a positive control treatment [200]. The negative control treatments were saline at 10% sample weight and sham, i.e., no substance added to mucus. Samples were divided into control (C) samples and RAO exacerbation (R) samples. C-samples (n = 21) were from control horses at any time point and from RAO-affected horses at time points 0 and 6 hours, since viscoelasticity does not change in control horses during environmental challenge and is normal at 0 and 6 hours in RAO-affected animals [169]. R-samples were from RAO-affected horses 24 or 48 hours after environmental challenge when the increase of mucus viscoelasticity occurs [169]. Large R-samples were carefully split for several measurements so that a sufficient number (n = 31) was

obtained for all treatments. Microrheological measurements were performed before and after 30 minutes incubation at 37°C with gelsolin, rhDNase, nacystelyn, saline or no treatment (sham). Samples were randomly assigned to treatments, which were performed in randomized order. As a further positive control, each treatment was also performed on a small number of cystic fibrosis (CF) mucus samples.

Statistical analysis and interpretation–All statistical analyses were performed using SPSS for Windows (SPSS Inc., 233 S. Wacker Drive 11th Floor, Chicago, IL 60606, USA). Paired one-tailed t-tests were used on viscoelasticity (log G*) and tan δ values of paired samples before and after treatments with gelsolin, rhDNase, nacystelyn, saline or no treatment (sham) to assess significance of mucolytic effects. For paired t-test analysis, samples were grouped together (C, R and CF) for negative (sham, saline) and positive (nacystelyn) control treatments, and grouped separately (C, R, CF) for endpoint treatments with gelsolin and rhDNase.

Results and discussion

Control treatments–The sham procedure on R, C and CF samples had no effect and showed very little variation (Table 1; Fig. 1A). Similarly, the saline control showed no significant change and only slightly more variation (Table 1; Fig. 1B). The positive control treatment nacystelyn showed a significant combined mucolytic effect on R, C and CF samples (Table 1). The overall decrease in viscoelasticity was more than 3-fold on a linear scale (Table 1). Fig. 1C shows that this was mainly due to a large effect on samples with high starting viscoelasticity. In contrast, samples that had a logG* of less than 2.5 dyn/cm^2 showed no effect. A paired t-test was therefore performed separately on control samples, since they are of inherently lower

viscoelasticity. The test confirmed that nacystelyn also had a significant effect on control samples (Table 1). This was expected since nacystelyn, a thiol reducing agent and mucolytic similar to N-acetyl-L-cysteine, indiscriminately breaks mucin disulfide bonds that make up the mucus gel network. Three CF samples each were used as positive controls for the mucolytic treatment with gelsolin and DNase. Although no statistical analysis was performed, Figs. 1F & I illustrate that the concentrations used had a strong mucolytic effect on CF samples in this study, as expected based on previous reports [199]. No effects on tan δ were observed with any of the control treatments (results not shown). The control treatments confirmed the validity of our mucolytic experiments.

Mucolytic effects of DNase on equine mucus samples–RhDNase treatments did not alter viscoelasticity of either mucus from RAO-affected horses in exacerbation or of control samples (Table 1). No effects on tan δ were observed with rhDNase in any of the sample groups (results not shown). Although the power of the test in the rhDNase/control sample group was limited due to the sample size, Figs. 1D & E do not show any trend of a mucolytic effect in either sample group. This is in agreement with Schatzmann et al (1973 [21]), who concluded that free DNA was not present in excess in RAO mucus compared with sputum from humans with chronic bronchitis. On the other hand, our results are in apparent conflict with those of Pietra et al. (1998 [172]) who found that RAO mucus, in contrast to that of horses affected by small airway inflammatory disease, had a high DNA content and was susceptible to the mucolytic effect of rhDNase. Although the methodology cannot be directly compared, Pietra et al. (1998 [172]) used a higher concentration of rhDNase, which could explain

the discrepancy in our findings. Our methodology was identical, however, with that previously used on CF-sputum [199], and we can therefore conclude that rhDNase concentrations that are effective on CF mucus do not work on RAO exacerbation or control equine samples. Thus, DNA fibers either do not play a similarly important rheological role in RAO mucus as in CF mucus, or, alternatively, they may be protected to a larger degree from mucolytic action. F-actin fibers have been shown to form an entangled network with DNA fibers [201], causing increases in viscoelasticity [171, 192], and also protecting DNA fibers from mucolytic action [199, 202].

Mucolytic effects of gelsolin on equine mucus samples–The gelsolin treatments offered evidence that F-actin may indeed play a role in RAO mucus rheology. As illustrated in Fig. 1G, gelsolin had a significant mucolytic effect on mucus from RAO horses in exacerbation, reducing viscoelasticity almost 3-fold (on a linear scale) to normal values. Unlike the nacystelyn control treatment, gelsolin did not change viscoelasticity of control samples (Table 1; Fig. 1H). This strong selective effect of gelsolin on RAO exacerbation mucus indicates that F-actin fibers may be involved in the unfavorable rheological changes we have observed in mucus from RAO-affected horses after stabling and hay exposure [169]. In order to better understand these mechanisms, however, further studies are needed. In particular, the effects of these mucolytic agents may have to be studied separately at different concentrations as well as in combination, since a synergistic effect of gelsolin and rhDNase has been demonstrated in CF sputum [199].

DNA/F-actin network- synergistic effects of DNase and gelsolin–Fluorescence microscopy of CF sputum has shown that F-actin and DNA co-

localize to form large aggregates in CF sputum and that DNA forms in vitro fiber aggregates only in the presence of actin [201]. These findings suggest that increases in mucus viscoelasticity may not simply be due to the presence of either F-actin and/or DNA fibres, but rather to an interaction of both fiber types to form large aggregates. The demonstration of a synergistic effect of gelsolin and rhDNase in CF sputum [199] provided further evidence for an interaction of the two degradation products. We propose that following neutrophilic inflammation in equine RAO, aggregates of F-actin and DNA fibers form in airway secretions and interact to cause the dramatic increases in mucus viscoelasticity observed during acute RAO exacerbation. Since the cost of the available gelsolin and rhDNase products are prohibitive in equine medicine, we cannot expect to derive direct profit for therapeutic application of these products in the near future. Nevertheless, investigations of mucolytic effects, such as performed in this preliminary study, can offer insight into the mechanisms of mucus rheological changes and accumulation of secretions in equine RAO.

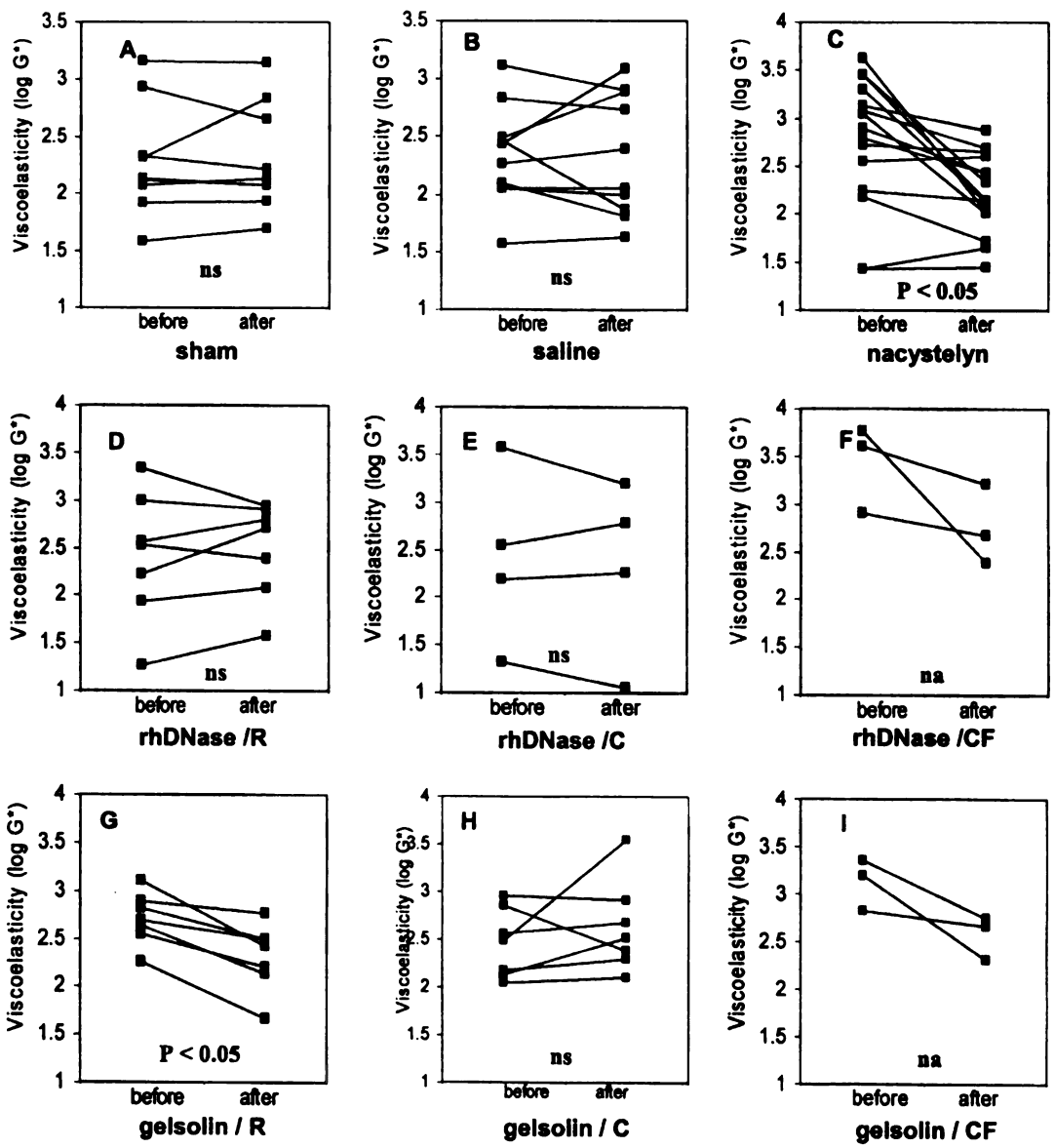
Conclusions–Our results indicate that mucus from RAO-affected horses in exacerbation is similarly susceptible to the action of gelsolin as CF sputum. Severing F-actin filaments reduced viscoelasticity of RAO exacerbation mucus to normal values. In contrast, a higher concentration of rhDNase than is effective in CF sputum may be necessary to achieve a mucolytic effect in RAO mucus [172]. Thus, F-actin fiber aggregates, possibly interacting with DNA fibres, appear to play a role in the unfavorable rheological changes of mucus from RAO-affected horses in exacerbation.

Table 6-1: Change of viscoelasticity (Δlog G*, dyn/cm^2) without any substance added (sham), after saline treatment and after mucolytic treatment with nacystelyn, gelsolin and rhDNase on RAO-exacerbation (R), control (C) and cystic fibrosis (CF) mucus samples.

treatment	sample groups used	number of samples	mean ± SD of change in viscoelasticity (Δlog G*, dyn/cm^2; P)
sham	R + C + CF	9 (6 + 1 + 2)	+0.02 ± 0.22; ns
saline	R + C + CF	10 (6 + 2 + 2)	-0.001 ± 0.35; ns
nacystelyn	R + C + CF	15 (5 + 7 + 3)	-0.53 ± 0.56; $P < 0.05$
nacystelyn	C	7	-0.48 ± 0.47; $P < 0.05$
gelsolin	R	7	-0.40 ± 0.21; $P < 0.01$
gelsolin	C	7	+0.18 ± 0.47; ns
gelsolin	CF	3	-0.56 ± 0.37; na
rhDNase	R	7	+0.08 ± 0.30; ns
rhDNase	C	4	+0.09 ± 0.28; ns
rhDNase	CF	3	-0.67 ± 0.61; na

$P > 0.1$ was considered not significant (ns). The 3 CF samples in gelsolin and DNase treatments were not statistically analyzed (na)

Fig. 6-1: Viscoelasticity (log G*) before and after treatment. Negative (sham, A and saline, B) and positive (nacystelyn, C) control treatments on RAO-exacerbation (R), control (C) and cystic fibrosis (CF) samples. RhDNase treatment on R (D), C (E) and CF (F) samples. Gelsolin treatment on R (G), C (H) and CF (I) samples. Significant ($P < 0.05$) and non-significant (ns) treatment effects are shown in the graphs. Effects of rhDNase and gelsolin on CF control samples (F and I, respectively) were not statistically analyzed (na).



CHAPTER 7

IDENTIFICATION OF EQUINE HOMOLOGUES OF GEL-FORMING MUCIN GENES AND SEMI-QUANTITATIVE MEASUREMENT OF OF eqMUC5AC AND eqMUC2 mRNA LEVELS IN POOLED SAMPLESA FROM DIFFERENT AIRWAY GENERATIONS OF RAO-AFFECTED AND HEALTHY HORSES

Gerber, V., Robinson, N.E., Venta, P.J., Rawson, J., Jefcoat, A.M., Hotchkiss, J.A. Mucin genes in horse airways: MUC5AC, but not MUC2 may play a role in recurrent airway obstruction. (2003). *Eq Vet Journal.*

In the first studies (chapter 2-4) I showed that airway mucus accumulation is increased 2-fold in RAO-affected in remission compared with healthy control horses and further increases more than 2-fold in RAO-affected horses during exacerbation. Further studies (chapter 5 and 6) showed that mucus viscoelasticity is normal in RAO-affected horses in remission, but increases 3-fold during exacerbation. This increase is due at least in part to breakdown products, in particular F-actin, from degenerate neutrophils.

Unfavorable rheological properties leading to decreased mucus clearance can only partly explain the increased mucus accumulation in RAO, however. Particularly during remission, but potentially also in exacerbation, increased production of mucus must also play a role. Based on findings in other species, any or all of the secreted, gel-forming mucins, MUC2, MUC5AC and/or MUC5B, could be expressed in horse airways and may be involved in increased mucin production in RAO.

In order to test the hypothesis that increased production of mucus is a cause of the observed increased mucus accumulation, I identified equine homologues of gel-forming mucins and investigated the regional distribution of mRNA-levels of equine mucin gene homologues at different airway generations.

Abstract

Increased mucin gene expression may be an important cause of mucus accumulation observed in recurrent airway obstruction (RAO)-affected horses. To date, however, no mucin gene sequences are available for the horse. Our goal was to identify equine homologues of gel-forming mucins and investigate their expression at different airway generations of healthy and RAO-affected horses. Two equine homologues were identified by cloning and sequencing fragments of equine (eq)MUC5AC and eqMUC2. Semi-quantitative RT-PCR on RNA from airways (generations 1, 5, 10, 15; small airways and parenchyma), stomach (glandular), and colon revealed that eqMUC5AC is expressed in equine stomach and in all of the airway samples. In contrast, eqMUC2 steady-state mRNA levels were detected in colon and very faintly in stomach, but not in airway tissue. EqMUC5AC expression was also compared to that of ZO-1, a tight junction protein, and eqMUC5AC/ZO-1 ratios were higher in RAO-affected compared to control horses at all airway generations. We conclude that eqMUC5AC is expressed in horse airways, but any expression of MUC2 is undetectable and unlikely to be of physiological consequence. EqMUC5AC up-regulation may be a primary mechanism responsible for mucus hypersecretion and accumulation in RAO.

Introduction

In equine recurrent airway obstruction (RAO), excess airway mucus may be due to hypersecretion and/or decreased clearance [72]. Based on histological observation of goblet cell hyperplasia, hypersecretion has long been associated with RAO (reviewed in [2, 3], and we found mucin glycoprotein alterations in bronchoalveolar lavage fluid (BALF) of RAO horses [54]. Furthermore, we showed that mucus accumulation in RAO can only partly be explained by decreased mucus clearability [169].

Therefore, we propose that up-regulation of specific mucin gene(s) may be a primary mechanism leading to hypersecretion of specific mucins, subsequent mucus accumulation, and possibly changes in glycosylation patterns. In order to investigate these hypotheses and their implications it is first necessary to characterize specific mucins and their expression in equine airways.

Three gel-forming mucins, which have been (partly) sequenced, are present in human and rodent airways: MUC2, MUC5AC and MUC5B [87, 93, 203-207]. Based on reactivity with polyclonal antibodies raised against the respective human mucins, it has been proposed that a homologue of MUC5AC is present in equine stomach [100], and that homologues of MUC5AC and MUC5B may be found in equine airway secretions [101, 102]. Since these findings are based on reactivity with antibodies that have been raised against human mucins, genetic identification of equine homologues of the main gel-forming mucins is a priority for the investigation of mucin hypersecretion in RAO.

To date, no equine mucin gene products have been genetically characterized. The aims of this study were, therefore, to identify equine homologues of human and rodent mucin genes, to develop RT-PCR-based assays to determine which gel-forming mucins are

expressed in airways of healthy and RAO-affected horses, and to investigate the regional distribution of mucin gene expression in the airway tree.

Materials and methods

Animals, tissue collection, and processing for RNA samples–Seven control horses (4 mares and 3 geldings; 8±7 years of age), without a history of and without clinical signs of chronic airway disease, and 5 horses (4 mares and 1 gelding; 17±6 years of age), with a history and clinical signs of RAO, were exposed to an indoor stable environment and hay-feeding for several days. Within an hour after euthanasia (by barbiturate overdose in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University), 1-2 cm segments of conducting airways were collected from airway generations (G) 1, 5, 10 and 15; from small airways (S) of approximately 1 mm diameter; and from parenchyma (P) without macroscopically visible airways. In addition, stomach and colon tissues of two of these animals were sampled. Immediately after excision, the tissues were submerged in 2-3 mL of TriReagent (Molecular Research Center, Inc., Cincinnati, OH) and stored at –80 °C. Total RNA was isolated from homogenized samples based on a previously described method [208] and resuspended in nuclease-free water containing rRNasin (40 units/100μL). RNA concentrations were determined with a fluorescent RNA-binding assay (RiboGreen; Molecular Probes, Eugene, OR), using a SpectraMax GEMINI spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA), and individual samples were diluted to a working concentration of 50ng/μL. For each experimental group (i.e., control and RAO horses) equal amounts of RNA from each individual were combined to

form pooled samples of each lung sample site (i.e., G1, G5, G10 and G15; S; and P). The pooling was necessary because the amount of RNA extracted from some of the individual samples was insufficient to run RT-PCR of MUC2, MUC5AC and ZO-1 in duplicate reactions. Electrophoresis of aliquots from each of the pooled samples was performed on a 2% agarose/formaldehyde gel (NuSieve 3:1; FMC Bioproducts, Rockland, ME) to insure the integrity of the RNA samples.

Identification of equine mucin genes eqMUC2 and eqMUC5AC–Adopting a strategy previously employed by Guzman *et al.* (1996 [209]), we used PCR primers (panspecies MUC primers; Table 1) which correspond to two octapeptide domains in the C-terminal region that are conserved between the human and rat MUC5AC and MUC2 homologues in order to detect homologous equine sequences. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed starting with a volume of 20 μL containing PCR buffer plus 5mM $MgCl_2$, 1 mM each dNTP, 10 units rRNasin, 125 ng oligo(dT) (Becton Dickinson, Bedford, MD), 100 ng total RNA from airway, stomach and colon samples, and 40 units of MMLV reverse transcriptase (Promega). All RNA samples were then incubated at 42°C for 15 min, followed by incubation at 95°C for 4 min (RT- reaction). A PCR master-mix (PCR buffer, 4 mM $MgCl_2$, 6 pmol each of panspecies MUC forward and reverse primers [Table 1], and 1.25 units *Taq* DNA polymerase [added after 85 °C pre-heating]) was heated to 85 °C for 5 min and then added to the cDNA (double-stranded complementary DNA from RT-reaction) samples, for a final volume of 50 μL. Samples were then immediately heated to 95°C for 3 min and then cycled 32 times at 95 °C for 30s, 56 °C for 60s, and 72 °C for 60s, after which an additional final extension step at 72 °C for 10 min was included. Electrophoresis of PCR

products (10 μL) was performed on 3 % agarose gels (NuSieve 3:1; FMC Bioproducts) stained with ethidium bromide. PCR products of expected size (Table 1) were excised from the gel and purified using the Wizard® PCR Preps DNA purification system (Promega) according to the manufacturer's instructions, and then cloned in One Shot™ competent E.coli (InvitroGen, Invitrogen Corporation, Carlsbad, California) using the pCR2.1TOPO™ (InvitroGen) vector according to the manufacturer's instructions. Plasmid DNA was purified using the Wizard® Plasmid Preps (Promega) and 1000 ng in 6μL of water was submitted to the MSU sequencing facility for sequencing with M13 (forward) and -21M13 (reverse) primers using Big Dye Terminator chemistry, and a 377 ABI Prism DNA Sequencer. Computer analysis of the sequences was performed using SeqWeb™ with the Wisconsin software package version 10.1™ (Genetics Computer Group (GCG), Inc. GCG, Madison, Wisconsin).

RT-PCR for eqMUC2 and eqMUC5AC and testing of tissue specificity–Based on the two consensus sequences (see results) primers specific for eqMUC2 and for eqMUC5AC (Table 1), respectively, were designed using Primer3 software[1]. RT-PCR (protocol as above; 36 cycles as determined in preliminary linear range-finding experiments) was performed on airway (individual samples and complete RAO and control pools of G 1, 5, 10, 15; S and P) stomach, and colon samples to detect steady-state levels of the respective mucins in the tissues. To control for DNA contamination, a RT- (reaction mix without MMLV reverse transcriptase, Promega) reaction was run for all samples, and all reactions were performed in duplicate. Duplicate PCR reactions were always run independently, but were based on the same RNA samples (i.e., the RNA was not independently extracted in duplicates). PCR products were run on a 3% agarose gel

(NuSieve 3:1), and, after ethidium bromide staining, documented with a Bio-Rad ChemiDoc image acquisition system and Quantity One (v4.0) quantitation software (Bio-Rad, Hercules, CA), running on a Dell OptiPlex GX1 computer. To confirm the specificity of the eqMUC2 and eqMUC5AC assays, PCR products of predicted size were excised from the gel and purified using the Wizard® PCR Preps DNA purification system (Promega). The PCR product DNA (~20 ng in 12 μL of water) was directly sequenced using big dye terminator reactions with eqMUC2 and eqMUC5AC forward and reverse primers (at 30 picomoles concentration), respectively, and sequences were compared to the eqMUC2 and eqMUC5AC consensus sequences using SeqWeb™ (GCG).

EqMUC5AC / ZO-1 ratio on pooled RAO and pooled control samples at different airway generations–The RNA of the airway RAO and control pools was analyzed to determine the steady-state levels of eqMUC5AC mRNA by semi-quantitative assessment of the amount of eqMUC5AC cDNA produced by RT-PCR in comparison to the amount of ZO-1 cDNA produced from the same sample. ZO-1, a tight junction gene, has been previously used as an internal standard to control for the proportion of epithelial cells in the samples [210]. RT-PCR using the eqMUC5AC primers (Table 1) was performed as described above. RT-PCR using a primer pair for ZO-1 (Table 1) was performed using the same reaction mixtures and protocols as described above, except for a different PCR profile: After the RT reaction, samples were immediately heated to 94°C for 3 min and then cycled 33 times at 94°C for 1 min, 51°C for 1 min, and 72 °C for 1 min, after which an additional final extension step at 72 °C for 10 min was included. The abundance of cDNA (proportional to the primer specific mRNA in the sample) was semi-quantitatively

assessed by densitometric analysis as described above. EqMUC5AC and ZO-1 RT-PCR products were all run on one gel, and band volume values (OD [optical density] * mm^2 [surface of product band]) were calculated from duplicates. Band volumes of the eqMUC5AC RT-PCR product were divided by the band volumes of the ZO-1 RT-PCR product bands. This eqMUC5AC / ZO-1 ratio was used as a semiquantitative measure of the steady-state level of eqMUC5AC mRNA in epithelium at each airway generation in control and RAO horses.

Results

Identification of equine mucin genes eqMUC2 and eqMUC5AC–RT-PCR amplification using the panspecies MUC primers (Table 1) of total RNA isolated from horse airways, stomach and colon samples yielded cDNA fragments of about 550bp. This is in agreement with the size range of cDNA fragments produced by these primers [209] from human, MUC5AC: 591 bp, MUC2: 540 bp, and from rat samples, rMuc2: 528 bp. Sequencing of these cloned cDNA fragments revealed two distinct mucin-like sequences. Colon sample clones showed a high degree of nucleotide sequence identity (73-77%) with MUC2 of other species (Table 2), indicating an equine homologue of MUC2 (proposed name eqMUC2; colon isolate GeneBank AF345996). The sequences from the stomach and airway samples showed a high percentage of nucleotide sequence identity (72-80%) with MUC5AC of other species (Table 2), indicating an equine homologue of MUC5AC (EdMUC5AC; GeneBank AF345995). A comparison of the eqMUC2 and eqMUC5AC fragments showed 57% nucleotide sequence identity.

RT-PCR for eqMUC2 and eqMUC5AC and testing of tissue specificity–RT-PCR product sizes using the primers developed for eqMUC2 and eqMUC5AC were about 190 and 250 bp, respectively (Fig. 1), as predicted from the primer design (Table 1), and direct sequencing confirmed the identity of the products with their respective target sequences. RT-PCR on airway, stomach and colon samples revealed that eqMUC5AC is expressed in equine stomach (clearly detectable in < 1 ng total RNA) and in all airway generations but is undetectable in colon samples up to the limit of specific (RT- up to 42 cycles) reactions. Fig. 1 shows the resulting bands from representative RT-PCR reactions at standard conditions, i.e., 36 cycles and 100 ng total RNA. EqMUC2 mRNA was detected in colon (strong band from <1 ng of total sample RNA) and very faintly in stomach (at standard conditions), but not in any of the airway tissue samples up to the limit of specific (RT- up to 40 cycles) reactions.

EqMUC5AC / ZO-1 on pooled control and pooled RAO airway samples–The product bands of eqMUC5AC and ZO-1 at each airway generation are shown in Fig. 2 along with a graphic representation of the ratios of their volumes. RT-PCR of ZO-1 produced two bands (~190 and 430 bp; Fig.2), as expected because of the alternative splicing of its mRNA transcripts [210]. Band volume values ($OD*mm^2$) of eqMUC5AC and ZO-1 (both bands) RT-PCR products are presented in Table 3. These values as well as the eqMUC5AC / ZO-1 ratios in Fig. 2 show that eqMUC5AC steady-state mRNA levels (both absolute and compared to ZO-1) were highest at airway generations G1, G10 and P in the pooled samples from healthy control horses as well as in RAO-affected animals. However, eqMUC5AC values were higher and ZO-1 values lower in RAO than in control pools at all airway generations. The ratio therefore accentuates the differences

between RAO and controls, which is small at G1, G16 and P, but pronounced at G5, G10 and S.

Discussion

We identified the first mucin gene sequences described in the horse: equine homologue fragments of MUC2 and MUC5AC (proposed names: eqMUC2 and eqMUC5AC, respectively). The interspecies nucleotide sequence identity (72-80%; Table 2) between equine, human, rat and mouse MUC2, and equine, human, pig, hamster, rat and mouse MUC5AC, respectively, was closer than between eqMUC2 and eqMUC5AC (57%). Our results confirm previous reports [209] that the cysteine-rich region in the terminal domain, which we identified and partly sequenced, is highly conserved across species. For instance, between the predicted equine MUC5AC and the known porcine MUC5AC amino-acid sequence 18 of 19 cysteines were conserved (results not shown). This underlines the functional significance of this region and, in particular, of the cysteines essential for oligomerization, i.e., gel-formation [211].

The detection of eqMUC2 mRNA steady-state levels in the colon samples (Fig. 1) is in accordance with the tissue distribution of this mucin in humans and rodents [212]. While there is evidence for a role of MUC2 in cystic fibrosis [99], and its expression in human airway cells in vitro can be induced by gram-negative bacteria, IL-9, as well as other agents and mediators [96, 213], data on the presence or absence of this mucin in healthy human airways is conflicting [91, 214]. We found no evidence for eqMUC2 expression in healthy equine airways or for a role for this mucin in RAO. In contrast, eqMUC5AC mRNA steady-state levels were detected in stomach and in each of the airway samples, but not in colon

(Fig. 1). This tissue distribution corresponds to that observed in humans and rodents [212]. Based on reactivity with a mix of polyclonal antibodies raised against human MUC5AC other investigators have proposed that a homologue of MUC5AC is present in equine airway secretions [101] and gastric surface mucus [100]. Our results support these findings, and the present evidence suggests that eqMUC5AC, but not eqMUC2 may be a major component of airway mucus in both healthy and diseased equine lungs. However, besides MUC5AC the other major component of human mucus identified is MUC5B [90, 91, 206, 215]. None of the cloned panspecies MUC RT-PCR products we analyzed to date were homologues of MUC5B (unpublished results). It is possible that this mucin was not expressed in the samples we investigated, since it is predominantly produced by submucosal glands, which are abundant in larger human bronchi, but very sparse in horse airways [70, 108]. However, based on polyclonal antibodies raised against human MUC5B, there is evidence that a homologue of this mucin is present in mucus from horse airways [102]. It seems therefore more likely that the panspecies MUC primers (Table 1) did not allow us to identify equine MUC5B homologue mRNA, even though it may have been present in the samples we studied. Further work will be needed to address the question of MUC5B expression in equine airways.

In a next step, we investigated eqMUC5AC steady-state levels at six different airway generations of pooled samples from control horses and from RAO-affected individuals. The intensity of eqMUC5AC product bands differed markedly between samples from different airway generations (Table 3; Fig. 2). Interestingly, a similar pattern of eqMUC5AC expression at different airway generations was observed both in control and RAO samples: generation 1 and 10 as well as the peripheral samples showed the strongest

expression of eqMUC5AC. At each airway generation, however, band volume values were higher in RAO than in controls. We did control quality of total pooled sample RNA, but the proportion of mRNA from inflammatory cells in the total sample RNA could have influenced these values. ZO-1, a tight junction gene, has been previously used to control for this variation [210]. We therefore compared the volume values of the eqMUC5AC RT-PCR product band to that of the ZO-1 RT-PCR product band at each airway generation pool sample. The eqMUC5 / ZO-1 ratio accentuated the differences between RAO and controls, which was small at G1, G16 and P, but very marked at G5, G10 and S (Fig. 2).

The limitations of our control vs. RAO comparison are clear: a semi-quantitative assay was used to evaluate differences between pooled samples of two groups with a considerable mean age difference. Future studies will have to address interindividual variability and the influence of age on mucin expression with quantitative RT-PCR, and the present results must be interpreted with caution. Also, the biological significance of the variation of MUC5AC expression in the different airway generations of both RAO and controls is unclear at this point. However, the consistency of the eqMUC5AC increase in RAO compared to control pools at all airway generations may indicate an important role of eqMUC5AC up-regulation in RAO. The large difference between RAO and controls in the peripheral airways (Table 3; Fig. 2) may be functionally important due to the very large proportion these small airways contribute to the total surface area of the airways.

In murine airways, Muc5AC mRNA is a marker of goblet cell metaplasia [216], and higher eqMUC5AC mRNA levels could simply be due to higher numbers of goblet cells per total epithelial cells. Specifically, an increase of goblet cells in small airways,

where we observed the largest difference between controls and RAO, has been reported by Kaup *et al.* (1990 [70, 217]). However, none of the studies reporting mucus epithelial hyper- and metaplasia in airways of RAO-affected horses (reviewed by Dixon, 1992 [2] and Robinson *et al.* 1996 [3]) have employed rigorous morphometric methods, and a better understanding of the histological correlate of the differences in mucin expression must await further studies. On the molecular level, various allergic, toxic and infectious stimuli, and the corresponding inflammatory mediators have now been shown to regulate expression of MUC2, MUC5AC and MUC5B [95, 96, 136, 145, 166, 170, 218-222]. Of particular comparative interest is the responsiveness of MUC5AC, which has been shown to be up-regulated by neutrophil- (i.e., oxidative stress, epidermal growth factor activation [151, 170], and allergen- (i.e., IL-4, IL-9 and IL-13; [144, 213, 216] induced mechanisms. Similar mechanisms may increase mucin mRNA levels in equine RAO, for which a Th2-type cytokine profile [158], severe neutrophilic airway inflammation associated with increased oxidative stress [152], as well as NF-κB activation [164] have been reported.

The RT-PCR assays we developed provide the first tools to investigate expression of specific mucin genes in horse airways, and the mucin gene sequences we identified may become the basis for development of equine mucin specific immunological assays. We conclude that of the two identified equine mucin genes, which show close similarities in the cysteine-rich flanking regions with their homologues in other species, eqMUC5AC but not eqMUC2 is expressed at detectable levels in horse airways. We further propose that increased eqMUC5AC production, in particular in the small pulmonary airways, is a potential primary mechanism responsible for mucus hypersecretion in equine RAO.

Table 7-1: Primer sequences, product size and references.

Name	Forward	Reverse	Size	Reference
Panspecies MUC	5' GGCCAGTGCGG CACTTGCACCAAC 3'	5' GCCCTCCGGACA GAAGCAGCCTTC 3'	~540bp[a] ~590bp[b] ~550bp[c,d]	[a,b] (14) [c,d]This paper
EqMUC2	5' ACTGTCCCCACTC CAGCTT 3'	5' GGTACCACACAG CTGTCGAA 3'	193bp[c]	This paper
EqMUC5AC	5' CTGTGTGTTTGAC CAGTGCC 3'	5' TTCTGTGATGGG TCCAGCTT 3'	247bp[d]	This paper
ZO-1	5' CATAGAATAGACT CCCCTGG 3'	5' CTGCTGGCTTGT TTCTCTAC 3'	~190bp[e] ~430bp[e]	(33)

RT-PCR product size in basepairs (bp) from respective primers on MUC2[a], MUC5AC[b], rMuc5AC[b], eqMUC2[c], eqMUC5AC[d] and the human and equine homologue of ZO-1[e] (two product bands due to alternative splicing).

Table 7-2: Nucleotide homology of (NS) of eqMUC2 and eqMUC5AC with other mucins.

Mucin cDNA	GenBank accession number	NS. identity and gaps
EqMUC2 fragment*		
MUC2 (human)	L21998	77% and 3%
Rmuc2 (rat)	M81920	73% and 3%
Muc2 (mouse)	AJ010437	73% and 5%
EqMUC5AC fragment*		
MUC5AC-like (pig)	AF054584	80% and 1%
MUC5AC (human)	Z48314	72% and 9%#
MUC5AC-like (hamster)	AF288468	72% and 3%
Rmuc5AC (rat)	U83139	72% and 2%
Muc5AC (mouse)	AJ010792	73% and 2%

*GenBank accession numbers: AF345996 for eqMUC2 and AF345995 for eqMUC5AC.
All gaps were consistent with gain or loss of complete codons; #gap percentage higher because of 18 amino-acid insertion in human MUC5AC sequence.

Table 7-3: EqMUC5AC and ZO-1 on pooled RAO and pooled control airway samples.

		Airway generation‡					
		G1	G5	G10	G16	S	P
EqMUC5AC*	Control	77.8	1.5	43.3	3.9	0.2	66.1
	RAO	82.2	34.7	70.8	6.9	66.4	83.8
ZO-1*	Control	75.6	13.5	84.3	53.7	17.3	69.6
	RAO	40.1	4.1	19.4	8.1	10.4	54.6

*Band volumes ($OD*mm^2$) of eqMUC5AC and ZO-1 (both bands) RT-PCR products, respectively (run all on one gel and calculated from duplicates). ‡Airway generations (G) 1, 5, 10 and 15; small airways (S) of approximately 1 mm diameter; and parenchyma (P) without macroscopically visible airways.

Fig. 7-1: Tissue specificity eqMUC2 and eqMUC5AC mRNA detected by RT-PCR. Representative samples from airway generation 1 (G1) from control and recurrent airway obstruction (RAO)- affected horses, as well as from stomach and colon tissue. Ethidium bromide stained PCR products were run on 3 % agarose gels.

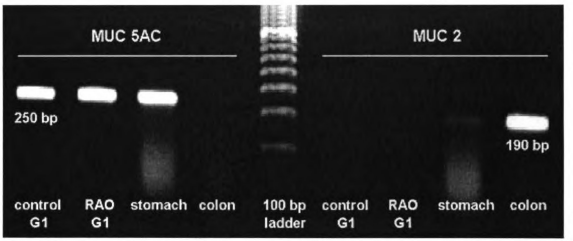
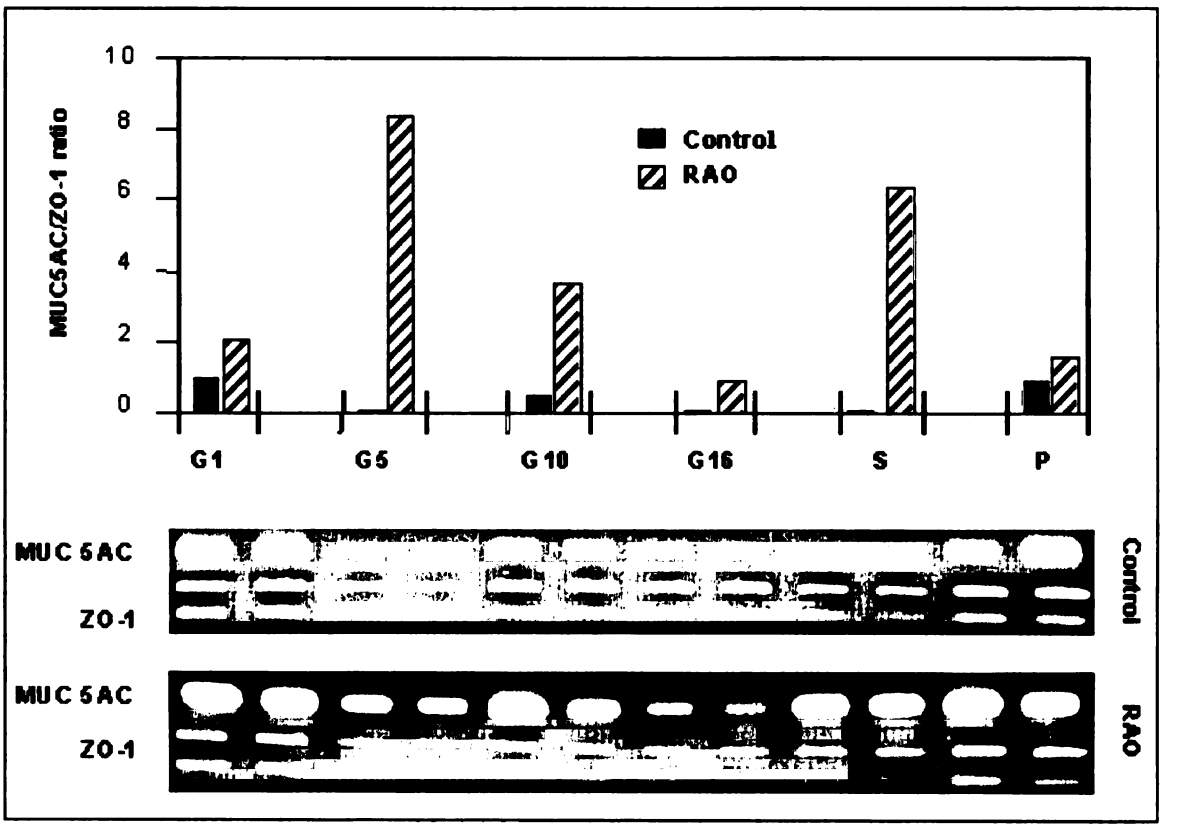


Fig. 7-2: EqMUC5AC and ZO-1 RT-PCR cDNA products at each airway generation and graphic representation of the ratios of the band volumes. All cDNA products were run on the same gel (ethidium bromide stained 3 % agarose), lanes shown were cut and pasted with graphic software. Pooled samples for control and recurrent airway obstruction (RAO)- affected horses from airway generations (G) 1, 5, 10 and 15; from small airways (S) of approximately 1 mm diameter; and from parenchyma (P) without macroscopically visible airways.



CHAPTER 8

MUC5AC mRNA LEVELS, BUT NOT INTRAEPITHELIAL MUCOSUBSTANCE, ARE ASSOCIATED WITH EQUINE CACC1-LIKE AND EGFR mRNA LEVELS AND WITH INTRALUMINAL NEUTROPHILS IN SMALL CARTILAGINOUS AIRWAYS OF RAO-AFFECTED AND CLINICALLY HEALTHY HORSES

I showed that unfavorable rheological properties (chapter 5 and 6) can only partly explain the increased airway mucus accumulation in RAO (chapter 2-4) and that equine(eq)MUC5AC, but not eqMUC2 may play a role in recurrent airway obstruction (chapter 7). Preliminary results indicated increased eqMUC5AC mRNA levels in RAO-affected compared to control horses at six different airway generations. These results were based on a semi-quantitative RT-PCR assay, however, and the airway generation samples had been pooled from several individuals per RAO and control group. I therefore developed a quantitative RT-PCR assay to accurately determine eqMUC5AC mRNA levels on individual samples and test the hypothesis that eqMUC5AC expression is increased in equine RAO.

Furthermore, I investigated the association of eqMUC5AC expression with stored intraepithelial mucosubstrance and with the expression of key signaling elements involved in the regulation of mucin expression and mucous cell metaplasia. MUC5AC is the main "signature" mucin in experimental and natural models of airway disease. Many different, Th-1 and -2 type as well as innate immunity, pathways converge on up-regulation of MUC5AC with subsequent mucous cell metaplasia and hypersecretion [136]. Epidermal

growth factor (EGF), its receptor, EGFR and homologues of the recently identified human calcium-activated chloride channel 1 (hCACC1) are key signaling molecules involved in these pathways, but have not been investigated in equine lung disease.

Abstract

We have previously found glycosylation alterations of mucin glycoproteins in recurrent airway obstruction (RAO)-affected horses that may be associated with increased secretion of equine(eq)MUC5AC. Epidermal growth factor (EGF), its receptor, EGFR and CACC1 are key signaling molecules involved in mucus cell meta- and hyperplasia and mucin gene expression, but have not been investigated in equine lung disease. We hypothesized that exposure to irritants and aeroallergens would lead to increased eqMUC5AC expression and stored mucosubstance in the epithelium of RAO-affected horses, associated with increased neutrophils and CACC1, EGFR and EGF mRNA levels. We therefore identified equine homologues of human(h)CACC1 and EGFR, and developed real-time quantitative RT-PCR to determine mRNA levels of eqMUC5AC, equine hCACC1-like, EGFR and EGF. These assays were performed on small cartilaginous airways from cranial left and right and caudal left and right lung lobes of 5 clinically healthy and 5 RAO-affected horses that had been exposed to indoor stable environment for 5 days before euthanasia. Morphometric measurements of volume densities of intraepithelial mucosubstances, as well as cytological differentiation of intraluminal inflammatory cells from the same airways were also performed. Neutrophil percentages were increased and macrophage percentages proportionally decreased in RAO-affected horses, but no significant differences of eqMUC5AC, equine hCACC1-like, EGFR and EGF mRNA levels or stored intraepithelial mucosubstance were found between RAO-affected and

control horses. Thus, the present results did not support the hypothesis that eqMUC5AC is up-regulated and intraepithelial mucosubstances are increased in RAO-affected compared to clinically healthy control horses. However, equine hCACC1-like and EGFR mRNA levels as well as neutrophil percentages were associated with eqMUC5AC mRNA levels in airways of RAO-affected and of clinically healthy horses. This suggests a role of these key signaling molecules in mucin regulation in airways of horses suffering from RAO as well as of horses with milder degrees of airway inflammation.

Introduction

Irritants (e.g., endotoxin) and allergens (e.g., mold spores are abundant in the environment of stabled horses [6, 7, 223] and induce clinical disease characterized by bronchospasm, intense neutrophilic airway inflammation and mucus accumulation in RAO-affected animals [3, 182], and milder neutrophilic inflammation without overt clinical signs in clinically normal horses [47, 174].

Similar irritants and allergens can increase mRNA steady state levels of specific mucin genes, in particular MUC5AC, and concomitant mucous epithelial hyper- and metaplasia in rodents [97, 222]. We found persistent alterations in glycosylation patterns of mucin glycoproteins in RAO-affected horses [54] that may be associated with increased secretion of MUC5AC. Therefore, we identified the equine homologues of MUC5AC and MUC2 [224]. A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay showed no detectable mRNA levels of equine(eq)MUC2, but robust expression of eqMUC5AC throughout the bronchial tree of RAO-affected and clinically

healthy horses. Semi-quantitative comparison on pooled samples suggested increased expression in RAO-affected animals.

MUC5AC can be up-regulated by neutrophil- [135, 175], and allergen- [118, 131] induced mechanisms. Several cytokines that are increased in equine RAO are known to up-regulate and activate epidermal growth factor receptor (EGFR) through epidermal growth factor (EGF) and other ligands. Neutrophils can upregulate EGFR expression through TNF-α and subsequently activate EGFR in a ligand-independent fashion through the release of oxidative radicals [134, 135]. Recently characterized calcium-activated chloride channels (CACC, in particular homologues of human(h)CACC1) are associated with increased stored intraepithelial mucosubstance, a measure of mucus cell meta- and hyperplasia, and increased MUC5AC expression in animal models of allergic airway disease as well as in asthmatic subjects [131-133]. Thus, EGFR and CACC are key signaling molecules involved in mucus cell meta- and hyperplasia and mucin gene expression, but have not been identified in the horse.

We hypothesized that exposure to irritants and aeroallergens would lead to increased eqMUC5AC expression and stored mucosubstance in the epithelium of RAO-affected horses compared with clinically normal control animals. We further proposed that eqMUC5AC mRNA levels and stored intraepithelial mucosubstance are associated with neutrophils and CACC1, EGFR and EGF mRNA levels.

The aims of this study were, therefore, to identify equine homologues of CACC and EGFR, and develop real-time quantitative RT-PCR to determine mRNA levels of eqMUC5AC, equine hCACC1-like, EGFR and EGF mRNA levels along with stored intraepithelial mucosubstance in airways of clinically healthy and RAO-affected horses, and

to investigate regional distribution of gene expression and stored intraepithelial mucosubstance.

Materials and methods

Animals, environmental exposure, euthanasia and sample collection–This study was approved by the All-University Committee for Animal Use and Care of Michigan State University. Five horses (2 mares and 3 geldings), 14 to 21 years old (mean ± s.e.m., 17.4 ± 3.4 years), with a previous diagnosis of RAO, served as the principal group. Five horses (3 mares and 2 geldings), aged between 18 and 28 years (mean ± s.e.m., 22.8 ± 4.1), and without clinical evidence of respiratory tract disease (i.e., respiratory distress, coughing), served as the control group. Horses were exposed to irritants and aeroallergens by stabling in stalls with straw bedding and feeding hay [6, 7, 223] for five days. Clinical signs of airway obstruction, i.e., increased breathing effort, were numerically scored as previously described [178]. In order to enter the study, control animals had to have a clinical score of ≤ 4 (3.1 ± 0.9; range 2-4) and RAO horses had to develop a clinical score of ≥ 5 (6.2 ± 1.2; range 5-8).

Samples for gene expression studies, morphometry and cytology were collected from the right cranial (a) and caudal (b) as well as the left cranial (c) and caudal (d) lung lobes within 30 minutes after euthanasia by barbiturate overdose. In each lung lobe 1cm length of a 2-3 mm diameter small cartilaginous conducting airway was excised for RNA isolation and immediately submerged in 2-3 mL of TriReagent (Molecular Research Center, Inc., Cincinnati, OH) and stored at –80 °C. For morphometric analysis an adjacent cross-section of the same airway was removed and submerged in fixative, and

for cytological analysis intraluminal secretions were sampled with a small cytology brush from the same airway distal to the excised tissues.

Cytological analysis of intraluminal secretions–Intraluminal secretions sampled by cytology brush were diluted in 500 µl of phosphate-buffered saline. Cell smears were made from the diluted samples with a cytocentrifuge and stained with Diff-Quick. Differential cell counts of macrophages, neutrophils, lymphocytes, eosinophils and mast cells were performed by counting 200 cells per sample using standard morphologic criteria under a light- microscope. The same person performed all cell scoring without knowledge of diagnoses. Differentials are expressed as percent of total cells.

Tissue preparation and morphometry of stored intraepithelial muco-substances–After fixation for 40 hours in 1% paraformaldehyde and 0.1% glutaraldehyde, the airway cross-sections were transferred to 30% EtOH and then embedded in glycol methacrylate, and 1-2 µm ultrathin sections were cut from the anterior surface. Sections were stained with Alcian Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS) to detect intraepithelial mucosubstances. To estimate the amount of the intraepithelial mucosubstances in the epithelium lining the axial airways, the volume density of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of AB/PAS stained mucosubstance was calculated from the automatically circumscribed perimeter of stained material using a Dell Optiplex GX260 computer and the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized

image of the basal lamina. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was estimated using a method described previously in detail [225]. The volume density of intraepithelial mucosubstances is expressed as nanoliters of intraepithelial mucosubstances per mm^2 of basal lamina. The same person performed the morphometrical analysis without knowledge of diagnoses.

Processing of RNA samples and reverse transcription of total RNA–Total RNA was isolated from homogenized samples based on the method of Chomczynski and Sacchi [208]. Isolated RNA pellets were resuspended in nuclease-free water and incubated with DNase solution [100 units rRNasin (Promega, Madison, WI), 100 mM DTT (Life Sciences Technology Inc., Grand Island, NY), and 10 units DNase I (Boehringer Mannheim, Indianapolis, IN) in 5X transcription buffer (Promega)] for 45 min. at 37°C. The RNA was extracted sequentially with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and precipitated with 10 M ammonium acetate and isopropanol. The pellet was washed with 75% ethanol, air dried, and resuspended in nuclease-free water containing rRNasin (40 units/100µl). Total RNA concentrations were determined using a PowerWave$_X$ spectrophotometer (Bio-Tek Instruments, Inc.). Reverse transcriptase reaction was performed in a volume of 20 µl containing 2 µl 10x buffer, 5 mM each dNTP, 1 unit/ µl rRNasin (SUPERase•In™; Ambion, Inc., 2130 Woodward Austin, TX 78744-1832, USA), 10 uM random hexamer primers, 2 µg total RNA, and 4 units of Omniscript reverse transcriptase (Qiagen, Inc., 28159 Avenue Stanford, Valencia, CA 91355, USA). All RNA samples in the 20 µl reaction mix were then incubated at 42°C for 60 min, followed by an incubation at 93°C for 5 min to inactivate the reverse transcriptase. To

control for DNA contamination, a RT- (reaction mix without reverse transcriptase) reaction was run for all samples. Preliminary quantitative PCR (protocol see below) showed DNA contamination in 5 of the samples. 2μg RNA of each of these 5 samples was again DNase-treated with DNA-free™ (Ambion) according to the manufacturer's instructions. Two of these 5 samples subsequently still showed DNA contamination and were eliminated from the study. Six other samples were eliminated from the study because insufficient amounts of total RNA were available.

Identification of equine homologous partial sequences of CACC1 and EGFR–Cross-species conserved regions were identified by comparison of human and mouse CACC1 (GeneBank [GenBank. Online. NCBI. http://www.ncbi.nlm.nih.gov)] accession numbers gi4585468 and gi3721911, respectively) and EGFR (gi29725608 and gi23271839, respectively) homologue nucleotide sequences, using the basic local alignment search tool (BLAST) 2 Sequences comparison [226] available through the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). Based on the identified conserved regions, cross-species consensus primers were developed. Primers were designed over less degenerate or mutable amino acids within regions of greatest nucleotide conservation. In most cases, the 3' end of each primer was designed to match the second codon position of an amino acid with low mutability to minimize the possibility of a mismatch at this position [227]. The second codon position for most amino acids is known to be less mutable than the first or third positions [228] (P.J. Venta personal communication). Melting temperatures of candidate primers were determined using algorithms available at the Virtual Genome Center website:

(http://alces.med.umn.edu)

Candidate primer pairs were tested by amplifying equine airway cDNA and sequencing PCR products. A PCR master-mix consisting of 25 µl 2x PCR buffer (including HotStarTaq DNA Polymerase and QuantiTect SYBR Green; Qiagen), 0.4 µM each of forward and reverse primers, and the cDNA samples (equivalent to 25 ng starting total RNA) for a final volume of 50 µl was heated to 95°C for 15 min and then cycled 50 times at 92 °C for 30s, variable annealing temperature based on primer melting temperatures for 60s, and 70 °C for 60s in a iCycler thermal cycler with iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, 1000 Alfred Nobel Drive Hercules, CA 94547). At the end of the PCR, the temperature was increased from 60 to 95 °C at a rate of 2 °C/min, and the fluorescence was measured every 15 s to construct the PCR product dissociation (melt) curve. A nontemplate control (NTC) was run with every assay, and all determinations were performed at least in duplicates to achieve reproducibility. When specific (single peak melt curve, primer-dimer melting temperature) amplification was observed, PCR samples were further analyzed. Electrophoresis of PCR products (10 µl) was performed on 3 % agarose gels (NuSieve 3:1; FMC Bioproducts) that were stained with ethidium bromide. PCR products were excised from the gel and purified using the Wizard® PCR Preps DNA purification system (Promega) according to the manufacturer's instructions, and then submitted for sequencing with specific forward or reverse primers using Big Dye Terminator chemistry, MJ Research DNA engine-PTC-200 for extension reactions and 377 ABI Prism DNA Sequencer to run gels after ethanol precipitation. Analysis of the sequences was performed using the standard nucleotide-nucleotide BLAST available through the NCBI web site (http://www.ncbi.nlm.nih.gov). The primer pair 5'-

ATTGGACAAAGAATTGTGTG-3' / 5'-ACAATTTCAGATCC ATCAGTT-3' (annealing temperature 52 °C) amplified a partial cDNA sequence of an equine hCACC1-like homologue and the primer pair 5'-GAGAGGAGAACTGCCAGAA-3' / 5'-GTAGCATTTATGGAGAGTG-3' (annealing temperature 47 °C) amplified a partial cDNA sequence of an equine EGFR homologue.

Selection of primers for quantitative RT-PCR–Using Primer3 software (Steve Rozen, Helen J. Skaletsky (1998) Primer3. Online. Code available at http://www-genome.wi.mit.edu/genome_software), primers with PCR products < 150 bp suitable for quantitative real time PCR , were designed based on an eqMUC5AC partial sequence (GeneBank accession number gi:19070191) we had previously identified [224], a published [229] complete cDNA sequence of equine EGF (gi:688071), a published [230] partial sequence of the equine 18s rRNA gene (gi:18369730) and the newly identified partial equine homologue sequences of EGFR and hCACC1 (see results). Quantitative real-time PCR (standardized protocol as above; annealing temperature 57 °C for all primers) was performed on airway (individual and pooled samples) cDNA. Primers were selected based on linear performance over a 5 log dilution range, 100 ng to 10pg starting RNA template per reaction. Specificity of amplified PCR products was monitored by melt curve analysis and confirmed by identification of a single band on gel electrophoresis, excision, purification and sequencing of RCR product as described above. Primers selected for real-time PCR are listed in Table 1. To assess if a primer pair amplifies genomic DNA, and what size the PCR product is, genomic DNA templates were also tested. To control for DNA contamination, RT- reactions were run for all samples and to detect primer-dimer formation negative (no template) reactions were

performed. Primers selected for real-time PCR based on product specificity, linear, efficient performance and absence of primer-dimer artifacts are listed in Table 1.

Relative quantitation of mRNA levels–Real-time PCR product accumulation was monitored using the intercalating dye, SYBR® Green I, which exhibits a higher fluorescence upon binding of double-stranded DNA. Relative gene expression was calculated using conditions at the early stages of PCR, when amplification was logarithmic and, thus, could be correlated to initial copy number of gene transcripts. The first 5-10 initial cycles were considered as a baseline or background in which no changes in fluorescence intensity occur. The level above this baseline, at which increments in fluorescence become detectable, is termed the threshold. For each target gene, the threshold was set at a predetermined value > 10 times the standard deviation of the mean baseline emission calculated for the baseline cycles. The iCycler iQ real-time PCR detection system (Bio-Rad Laboratories) software determined the cycle number when a reaction reached the threshold. This value, termed the 'cycle threshold' (C_t), appears during the exponential phase of the PCR and is inversely proportional to the initial number of template molecules in the sample. Standard curves (100ng, 50ng, 10ng, 5ng and 1ng starting RNA/reaction) of cDNA pooled from 5 samples were run in triplicate on the same plate as the unknown samples for each target gene. These standard curves displayed a linear relationship between Ct values and the logarithm of the starting RNA concentrations calculated using linear regression analysis. All unknown samples were within the C_t range of the standard curves for all target genes. Amount of target in each unknown sample was determined by interpolation from the standard curve of the target gene relative to the amount of ribosomal RNA in the same sample calculated from the

standard curve for 18s rRNA. This unit-less ratio was then expressed as an x-fold difference compared to the sample that showed the lowest levels for all target genes. Coefficients of variance of within-run and between-run assays were less than 5% for all assays. The correlation coefficient of the RNA concentrations and Ct values was >0.95 for all standard curves. The same person performed all relative quantitative RT-PCR without knowledge of diagnoses.

Statistical analysis–All statistical analyses were performed using a statistical software program (SAS[2] for Windows95, version 8.02). Dependent measures were analyzed by repeated measures ANOVA using the general linear model. Percentages of macrophages, neutrophils, lymphocytes, eosinophils and mast cells; volume density of intraepithelial mucosubstances; and eqMUC5AC, equine hCACC1-like, EGFR and EGF mRNA levels were the dependent measures of interest. Independent factors in the model included two levels (RAO-affected and control horses) of the between subjects factor, group, and four levels (right cranial lobe {a}, left cranial lobe {b}, right caudal lobe {c} and left caudal lobe {d}) of the repeated measure, location. Partial correlation analysis was used to probe the relationships of the two endpoint measures, eqMUC5AC mRNA levels and volume density of intraepithelial mucosubstances with each other, with neutrophil percentages and mRNA levels of equine hCACC1-like, EGFR and EGF. Partial variables included the 2 levels of group and 10 levels of individuals. For all analyses, significant differences were declared if $P < 0.05$. All data are herein expressed as the means ± SD.

Results

Cytological analysis of intraluminal secretions–A complete set of 40 airway secretion samples was available for cytological evaluation. Summary data are presented in Table 3. There was a significant effect of group on macrophage and neutrophil percentages. Neutrophils were more than double (66 ± 21 %) in RAO-affected horses than in control animals (29 ± 29 %). Macrophages (20 ± 12 % vs. 44 ± 23 %) and all other cell types were proportionally decreased in RAO horses compared to the control group. For lymphocyte, eosinophil, and mast cell counts, there were no main or interaction effects. Localization (cranial vs. caudal, left vs. right lobes) had no effect on any of the cytological variables.

Morphometry of stored intraepithelial mucosubstances–Thirty-seven airways could be morphometrically evaluated, in three airways volume density could not be measured due to excessive artefacts on the histological slides. The volume density of intraepithelial mucosubstances showed no significant differences between groups or between lung lobes. Means were similar between RAO-affected and control horses (Table 1), but individual measurements varied considerable between and also within horses (Fig. 1A). Within the same horse, for example horse V, Fig. 1A) some airways may appear to store mucosubstance in rounded goblet cells (lobe c, Fig. 1A and B), while other airways showed goblet cells expressing mucus (lobe a, Fig. 1A and C), or were almost completely devoid of intraepithelial mucosubstance (lobe b, Fig. 1A). The volume density of intraepithelial mucosubstances was not significantly correlated with neutrophils or mRNA levels of eqMUC5AC, equine hCACC1-like, EGFR or EGF.

Identification of equine hCACC1-like and EGFR homologues–RT-PCR amplification using the cross-species CACC1 primer pair (see methods) yielded a cDNA fragment of about 293bp. Sequencing of this cDNA fragment revealed a high degree of nucleotide sequence identity (257/298bp nucleotides; 86%) with human CACC1 (=hCLCA1; gi:4502864), indicating an equine CACC1-like gene cDNA fragment. Other similarities were found with a porcine chloride channel protein (194/234bp, 82%; gi:6002645), with mouse(m)CLCA3 (=gob-5, =mCACC1; 85/96bp, 88%; gi:8567335 and gi:3721911) and with rat mCACC2/3-like (144/176bp, 81%; gi:27729602). RT-PCR amplification using the cross-species EGFR primer pair (see methods) yielded a cDNA fragment of about 406bp. Sequencing of this cDNA fragment revealed a high degree of nucleotide sequence identity (366/413bp, 88%) with human EGFR (gi:29725608), mouse EGFR (342/391bp, 87%; gi:458123), rat EGFR (337/385bp, 87%; gi:25742616), porcine EGFR (343/394bp, 87%; gi:21913175), rabbit EGFR (158/176bp, 89%; gi:13173350) and chicken EGFR (288/358bp, 80%; gi:211740), indicating partial equine EGFR homologue cDNA.

Relative quantitative RT-PCR–Thirty-four airway RNA samples were available for quantitative RT-PCR of eqMUC5AC, equine hCACC1-like, EGFR and EGF. None of these target genes showed significant differences between groups (RAO-affected and control horses) or between lung lobes (a, b, c and d). EqMUC5AC showed very large variation between horses but relative good agreement between lung lobe samples within horses (Fig. 3A). Mean EqMUC5AC mRNA levels were higher in the control group (Table 1), although not significantly, due mainly to the caudal lobe samples in one control horse (ii, b and d). Mean equine hCACC1-like mRNA levels were higher in the

RAO group (Table 1), but again not significantly because of large variations between horses with relatively consistent levels within individuals (Fig. 3B). Similarly, for EGFR mRNA levels it was again horse I of the RAO-group that had the highest expression levels (Fig. 3C), but overall there was less difference between horses than for equine hCACC1-like. EGF showed somewhat less consistency within horses than the other gene targets, but the least apparent differences between individuals (Fig. 3D). Pearson correlation analysis on means of all lobes per individual showed significant correlations between eqMUC5AC mRNA levels and equine hCACC1-like (partial correlation coefficient r = 0.47, P= 0.002; Fig. 3A) and EGFR (r = 0.3471, P = 0.028; Fig. 3B) mRNA levels as well as neutrophil percentages (r = 0.34, P = 0.032; Fig. 3C), but no significant association was found with volume density of intraepithelial mucosubstance and mRNA levels of EGF.

Discussion

In the present study, mRNA levels of eqMUC5AC, but not intraepithelial mucosubstance were correlated with equine hCACC1-like and EGFR mRNA levels and with neutrophil percentages in small cartilaginous airways of RAO-affected and control horses. No significant differences of eqMUC5AC, equine hCACC1-like, EGFR and EGF mRNA levels or stored intraepithelial mucosubstance were found between RAO-affected and control horses, however.

Based on our previous studies [51, 179, 182, 231] using endoscopic observation to quantify visible mucus accumulations in the airways, we have estimated that 2-8 ml per hour of endoscopically visible mucus are transported along the trachea in clinically

healthy horses. Compared with healthy animals, in RAO-affected animals these volumes can be extrapolated to 2-3 times more per hour at pasture and 3-10 times more after exposure to the dust-laden stable environment. These increased mucus accumulations are associated with the neutrophilic inflammation in the airways [51, 179].

Overall, mucus accumulation in RAO-affected horses is likely due to a combination of factors, which all may be related to airway neutrophilia, a hallmark of RAO. Neutrophils can potentially cause to both mucus hypersecretion and decreased clearance. Since we found that decreased clearability of mucus can only partly explain the increased accumulations in RAO [169], we hypothesized that up-regulation of production at the level of mucin-gene expression may contribute to the mucus accumulation. This would be in accordance with the often described, but never proven epithelial mucus cell meta- and hyperplasia in RAO [2, 3]. Indeed, we found persistent alterations in glycosylation patterns of mucin glycoproteins in RAO-affected horses [54] that may be associated with increased secretion of MUC5AC. Therefore, we identified equine homologues of MUC5AC and MUC2 [224]. A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay showed no detectable mRNA levels of equine(eq)MUC2, but robust expression of eqMUC5AC throughout the bronchial tree of RAO-affected and clinically healthy horses. Semi-quantitative comparison on pooled samples suggested increased expression in RAO-affected animals.

In this study, we had therefore hypothesized that exposure to irritants and aeroallergens would lead to increased eqMUC5AC expression and stored mucosubstance in the epithelium of RAO-affected horses compared with clinically normal control animals,

and further proposed that eqMUC5AC mRNA levels and stored intraepithelial mucosubstance are associated with neutrophils and CACC1, EGFR and EGF mRNA levels.

The first part of this hypothesis was clearly not supported by our present findings. Only neutrophil and macrophage percentages were significantly different between disease groups. This is not surprising since neutrophils invade the airways of RAO-affected horses within 6 hours of exposure [14] to high dust loads, in particular endotoxin and mould allergens from hay feeding and straw bedding [6, 8, 156, 232]. The experimental protocol of five days of exposure was chosen because we expected maximal differences at this time point. Within a day of exposure endoscopically visible airway mucus accumulation approximately doubles in RAO-affected animals, but remains unchanged in clinically healthy controls [182]. Increased accumulation scores are not specific for RAO, however, and can be observed in various equine lower airway diseases [5]. Even in clinically healthy sport horses that perform well there is large variation and markedly increased airway mucus accumulation is frequently observed [231].

Furthermore, even though maximal clinical signs, mainly reflective of bronchospasm [3, 178], can be consistently reproduced in RAO-affected horses exposed for several days [3], clinical scores in this study were not very different between RAO-affected (6.2 ± 1.2; range 5-8; cut-off RAO: 5 or more [178]) and clinically healthy horses (3.1 ± 0.9; range 2-4; cut off clinically normal: up to 4 [178]). However, animals with normal clinical scores can have subclinical airway obstruction [178]. This and the fact that mean (Table 2) and individual (results not shown) neutrophil percentages in control horses were well above proposed normal values in small airway secretions of horses [12] indicate that after the 5 days of exposure there was subclinical, but significant

inflammatory airway disease among the animals in the control group. This is in accordance with the previously observed high incidence and large variability of neutrophilic inflammation and mucus accumulation in airways of stabled, clinically healthy horses [47, 182, 231] and may explain the lack of significant differences of intraepithelial mucosubstance and eqMUC5AC mRNA levels between the disease groups in this study. Methodological differences could also have contributed to the discrepancy between our earlier preliminary [224] and the present results. In our earlier report we had used a semi-quantitative RT-PCR to test samples that were pooled by disease group. The present results show that large (> 100x) differences in eqMUC5AC mRNA levels between individuals (Fig. 1A) could have disproportionately influenced the previous results based on the semi-quantitative comparison of the pooled samples.

Mucus epithelial hyperplasia and metaplasia in airways of RAO-affected horses have been reported in numerous studies (reviewed by Dixon [7] and Robinson et al. [29]). An increase in goblet cells specifically in small conducting airways, which we investigated in this study, has been reported [70, 217]. None of these reports are based on quantitative measurements, however. In contrast, the present study is the first to employ rigorous morphometric methods. There are two explanations for the lack of a difference observed in this study. RAO-affected horses may have low, (i.e., normal) amounts of stored intraepithelial mucosubstance. Alternatively, the clinically healthy control horses, which had evidence of significant airway inflammation, had mucus epithelial hyperplasia and metaplasia. Comparison with volume density of intraepithelial mucosubstance obtained by the same morphometric method in proximal and distal axial airways of rats shows that means in horses of both disease groups are more than 10-fold higher than in untreated

rats, and are in the range of values found in rats exposed to ozone and endotoxin. Even though such cross-species comparisons, especially with rodents, must be interpreted very cautiously, this supports the alternative explanation, that clinically healthy control horses have significant mucus epithelial hyperplasia and metaplasia. Ideally, we should have included a control group of clinically healthy horses that have never been exposed to stable environment, but such age-matched controls are virtually impossible to find in the Northern hemisphere.

Considerable variation of intraepithelial mucosubstances was noted not only between, but also within horses (Fig. 1A). Statistical analysis showed neither regularity (e.g., cranial vs. caudal lobes) of this variation, nor any association with neutrophil percentages, eqMUc5AC, equine hCACC1-like, EGFR or EGF mRNA levels. Subjective observations suggested that within the same horse different airways appeared to be in different stages of mucosubstance storage (Fig. 1B) or collective release (Fig. 1C). We have not quantified this observation and can only speculate that consistency within airways, but heterogeneity within the lung of these storage and release stages may explain the variations in intraepithelial mucosubstances. In absence of a better biological explanation, artefactual variation, i.e., release of mucosubstance post mortem due to traumatic handling, must also be considered. We can only stress that all samples were dissected and collected with the utmost care and immediately placed in fixative.

In contrast to intraepithelial mucosubstances, the relative, quantitative assessment of eqMUC5AC, equine hCACC1-like, EGFR or EGF mRNA levels showed remarkable consistency within individuals (Fig. 2A-D), indicating that the underlying pathogenesis is a diffuse process involving the entire organ. Conversely, differences between horses were

considerable. These differences reflect the variability in the degrees of mucus accumulation observed in RAO-affected and particularly also in clinically healthy horses [182, 231]. While the present results cannot explain the increased mucus in RAO airways, they may indicate that in some horses, RAO-affected, but also clinically healthy with milder degrees of airway disease, eqMUC5AC mRNA up-regulation may contribute to mucus hypersecretion. An important link, which has been demonstrated in other species, but not in the horse, is the demonstration that mucin mRNA levels are associated with mucin protein translation and finally secretion.

Perhaps the most exciting results of this study were associations of eqMUC5AC mRNA levels with equine hCACC1-like and EGFR mRNA levels and with neutrophil percentages, but not with EGF mRNA levels. Mindful of the dangers of drawing conclusions from multiple correlations in a limited data set, these associations connect mucin gene expression with key signaling elements in the pathogenesis of airway mucus cell hyperplasia and hypersecretion.

MUC5AC can be up-regulated by neutrophil- [135, 175], and allergen- [118, 131] induced mechanisms. Several cytokines that are increased in equine RAO are known to up-regulate and activate EGFR through EGF and other ligands. Neutrophils can upregulate EGFR expression through TNF-α and subsequently activate EGFR in a ligand-independent fashion through the release of oxidative radicals [134, 135]. Interestingly, residual lung function deficits and persistent neutrophilic inflammation in RAO remission and are highly correlated with increased NF-κB activity and ICAM-1 expression of epithelial cells [164]. NF-κB is an important intracellular signaling element that is involved in mucin gene up-regulation in vitro [96]. Sustained NF-κB activation in

equine RAO is driven by granulocytes and mediated by IL-1β and TNF-α [165], both of these cytokines increase MUC5AC mRNA levels in vitro [166]. The recently characterized calcium-activated chloride channel (CACC1) is associated with increased stored intraepithelial mucosubstance, a measure of mucus cell meta- and hyperplasia, and increased MUC5AC expression in animal models of allergic airway disease and in asthmatic subjects [131-133]. Our findings indicate that these pathways may be implicated in the pathogenesis of airway mucus accumulation in equine RAO and in milder forms of lower airway disease. Due to the large variations between individuals, larger numbers of horses need to be investigated.

In conclusion, the present results did not support the hypothesis that eqMUC5AC is up-regulated and intraepithelial mucosubstances are increased in RAO-affected compared to clinically healthy control horses. However, we have identified equine homologues EGFR and hCACC1, which important regulators of mucin gene expression and mucus cell changes in the airways. Equine hCACC1-like and EGFR mRNA levels as well as neutrophil percentages were associated with eqMUC5AC mRNA levels in airways of RAO-affected and of clinically healthy horses. This suggests a role of these key signaling molecules in combination with neutrophilic inflammation in mucin regulation in airways of horses suffering from RAO as well as of horses with milder degrees of airway inflammation.

Table 8-1: Real-time PCR primer sequences (5' to 3') and predicted product size in basepairs

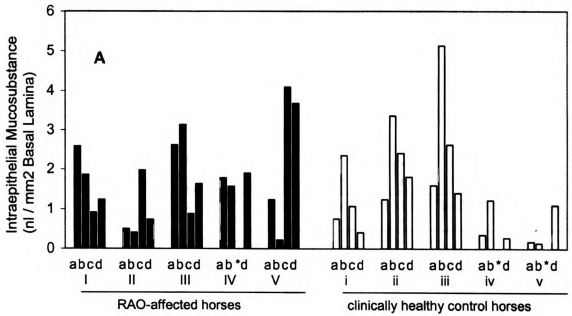
Name	Forward	Reverse	Size
MUC5AC	CTGCCTCTTGCCCACCTT	GTGGCACTGGTCAAACACAC	120bp
EGF	CCCAGTCCTATGACGGGTACT	CTCAAGTCCTGGTGCTGACA	124bp
CACC1	CCGCCTTAAACGACTGACTC	TCCCTATCAGTGCCACCTTT	144bp
EGFR	GATGGACGTCAACCCAGATG	TTCCTCCACCTCGTAGCTGT	133bp
18S rRNA	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA	123bp

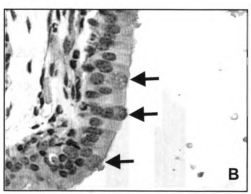
Table 8-2: Airway secretion cytology, intraepithelial mucosubstance and gene expression in RAO-affected and control horses: Number of observations, mean values ± SD for percentages (%) of macrophages, neutrophils, lymphocytes, mast cells and eosinophils, volume density (Vs, nl / mm^2 basal lamina) of intraepithelial mucosubstance (IMS), and relative mRNA levels of eqMUC5AC, equine hCACC1-like, EGFR and EGF.

	RAO-affected horses (n = 5)		Control horses (n = 5)	
	Number of Observations	Mean ± SD	Number of Observations	Mean ± SD
Macrophages	20	19.8 ± 11.9*	20	44.4 ± 22.5
Neutrophils	20	65.7 ± 21.3*	20	28.7 ± 28.7
Lymphocytes	20	12.1 ± 10.2	20	16.4 ± 10.6
Mast Cells	20	0.9 ± 1.7	20	2.6 ± 3.9
Eosinophils	20	1.3 ± 2.7	20	3.6 ± 7.0
Vs of IMS	19	1.7 ± 1.1	18	1.5 ± 1.3
eqMUC5AC	17	46.9 ± 42.9	17	64.4 ± 76.5
CACC1	17	115.8 ± 84.9	17	47.9 ± 36.7
EGFR	17	17.6 ± 16.8	17	10.1 ± 8.2
EGF	17	12.4 ± 7.7	17	9.9 ± 5.4

*significantly different ($P < 0.05$) from control group.

Fig. 8-1: Volume density of intraepithelial mucosubstance (A) in lung lobes a, b, c and d of 5 RAO-affected (I-V; black bars) and 5 clinically healthy control (i-v; white bars) horses. *In three airways volume density could not be measured due to excessive artefacts. Volume densities did not differ between groups or between lung lobes. Within a horse some airways could appear to store mucosubstance in rounded goblet cells (B; horse V, lobe c), while others showed goblet cells expressing mucus (C; horse V, lobe a), or were almost completely devoid of mucosubstance (horse V, lobe b).





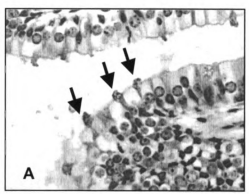
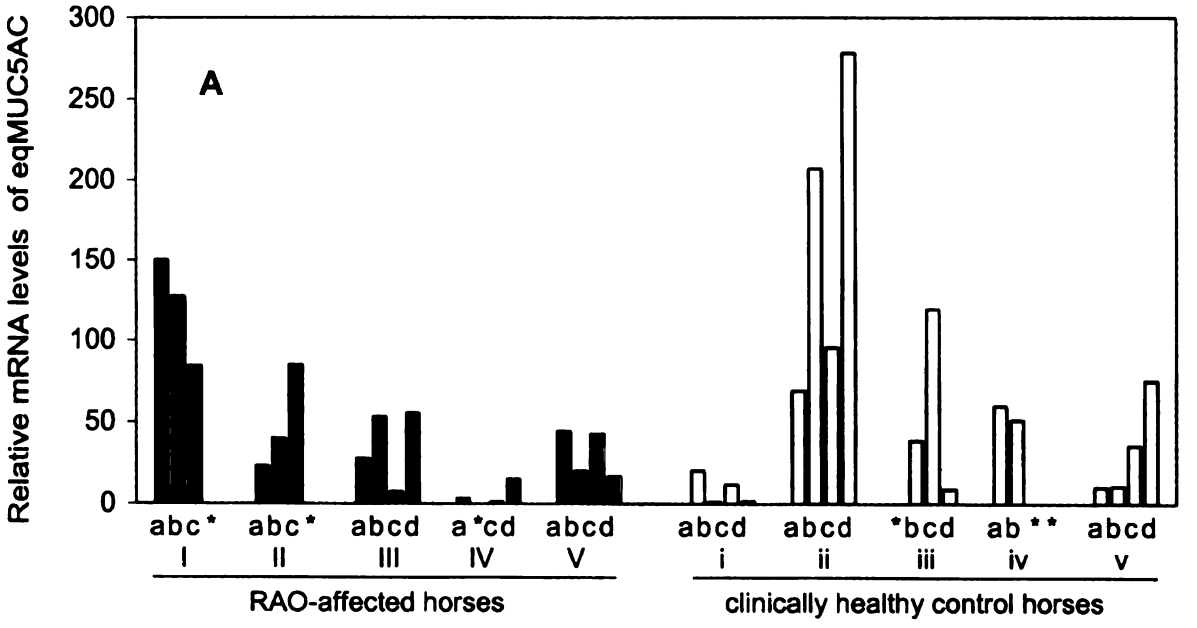


Fig. 8-2: Distribution of relative mRNA levels of eqMUC5AC(A), CACC1(B), EGFR (C) and EGF (D) in the 4 lung lobes (a, b, c and d) of 5 RAO-affected (black bars) and 5 clinically healthy control (white bars) horses. All results are expressed as relative x-fold levels of mRNA compared with the sample that was lowest for all target genes, Ivc, which is set as 1-fold. *Six RNA samples were not available for measurements.



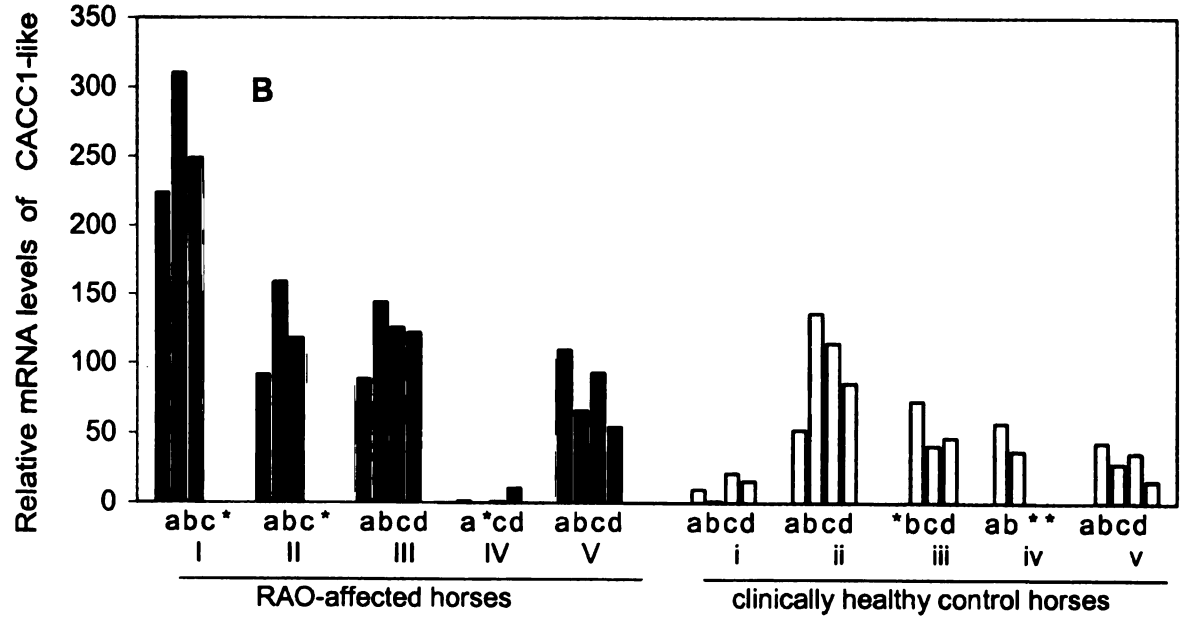
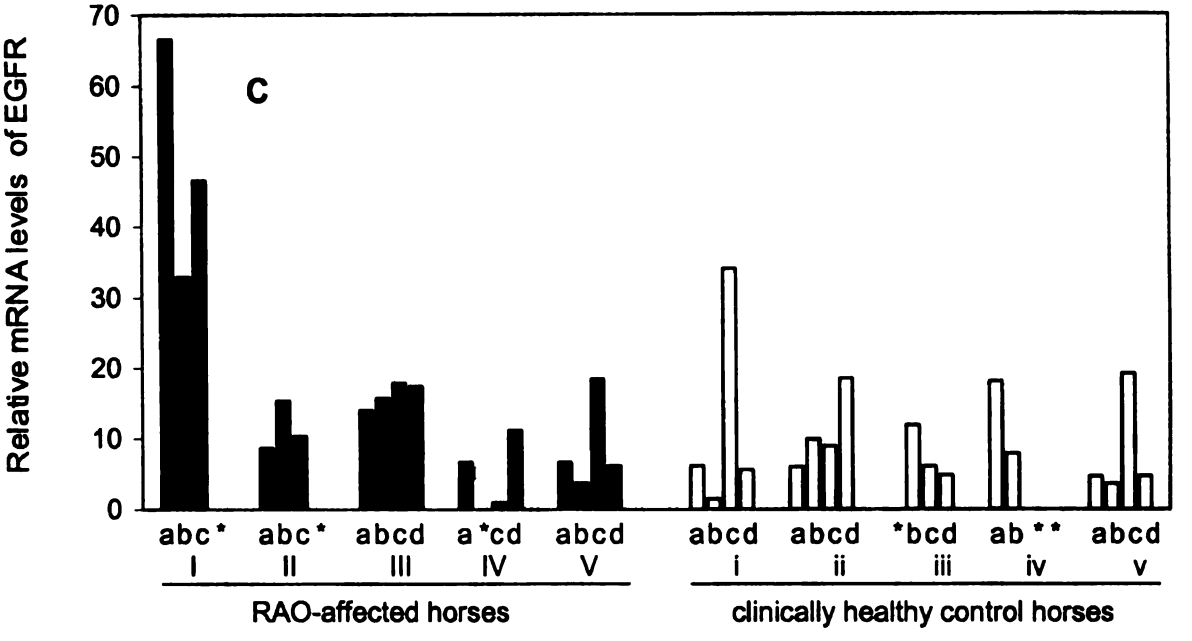


Fig. 8-2 (con't)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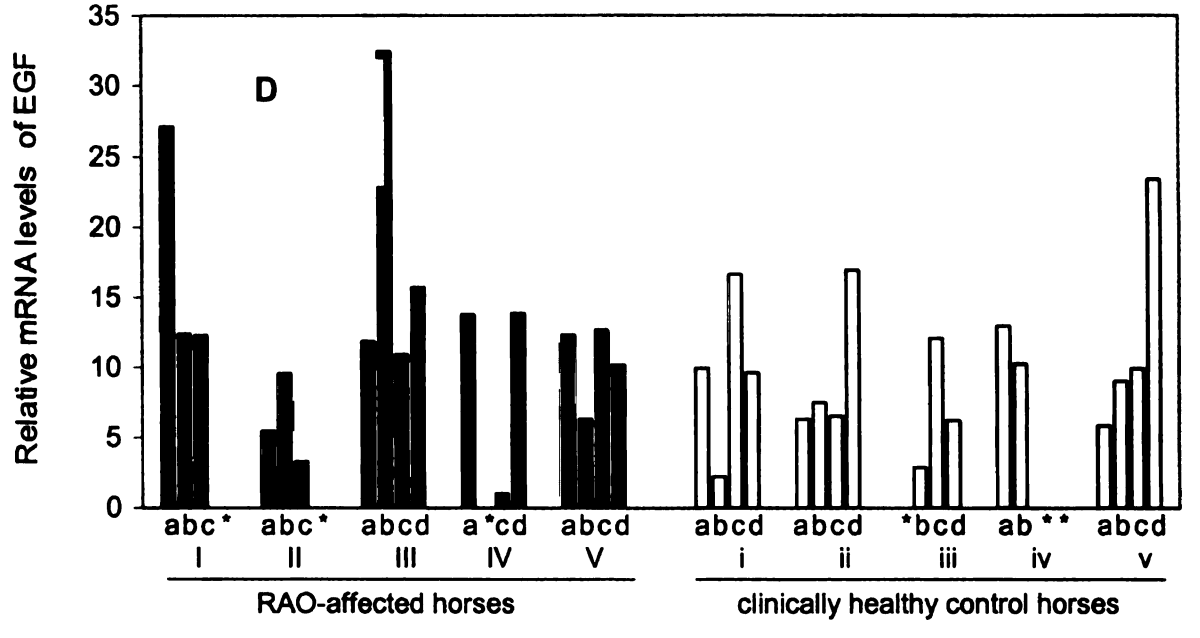
RAO-affected horses
clinically healthy control horses


D
Relative mRNA levels of EGF
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ab**
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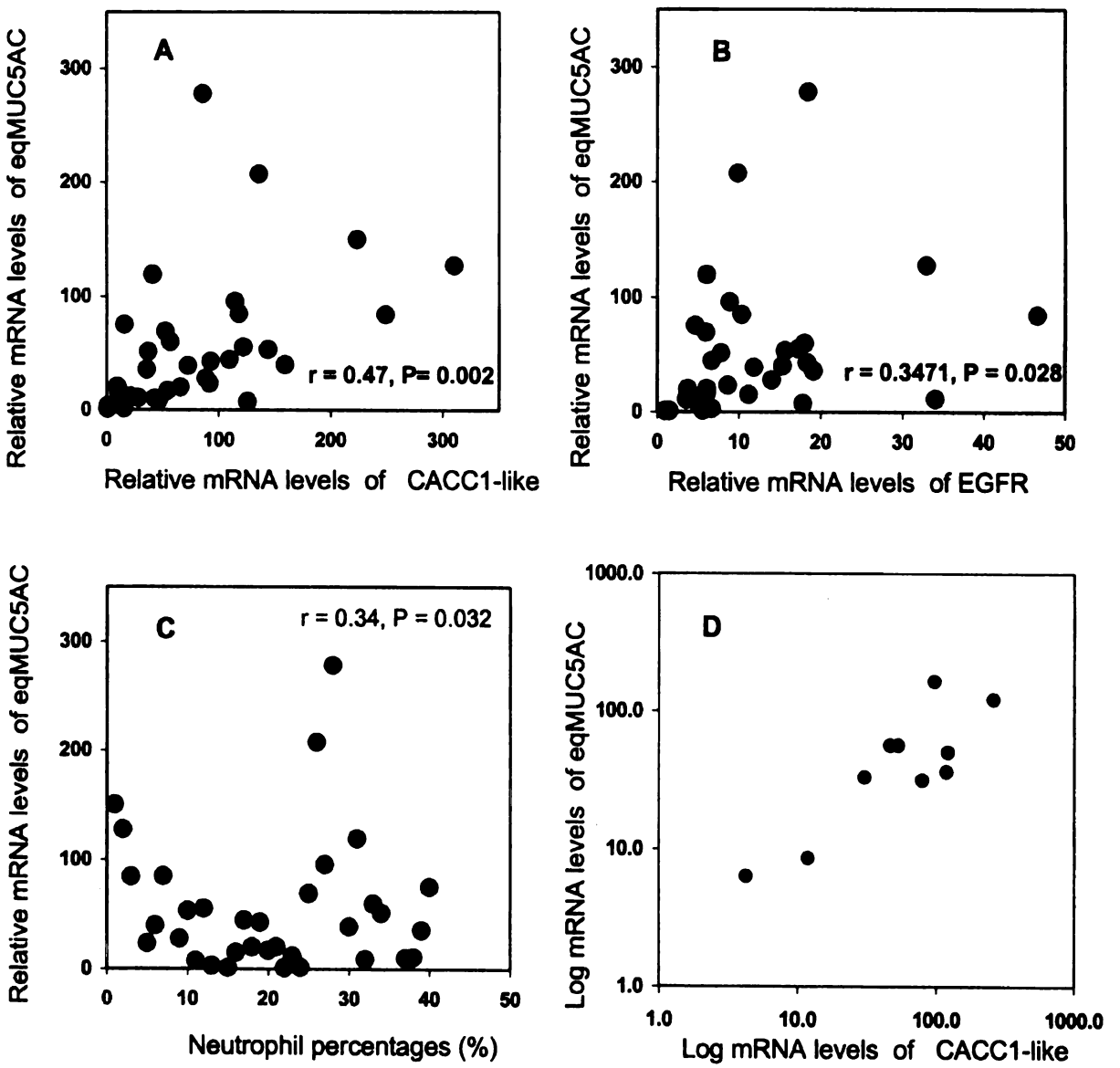
v
RAO-affected horses
clinically healthy control horses

Figure 8-3: Partial correlation analysis (r = partial correlation coefficient): significant correlations between eqMUC5AC mRNA levels and equine hCACC1-like (A) and EGFR (B) mRNA levels as well as neutrophil percentages (C). Fig. D illustrates the loglinear relationship between mean (a, b, c, d lobes) eqMUC5AC mRNA levels and equine hCACC1-like.



CHAPTER 9

SUMMARY, CONCLUSIONS AND OUTLOOK

I developed and tested three major hypotheses during my thesis project. In the following, I will discuss the results in the context of previous findings in equine lower airway disease and relevant comparative aspects of the literature.

Background

A literature review showed that increased airway mucus accumulation is a clinically significant problem in equine lower airway disease, both in recurrent airway obstruction (RAO) and, particularly in racehorses, in the milder inflammatory airway disease (IAD). However, there is no critically validated tool to quantify mucus accumulation in equine RAO and IAD, and, furthermore, the causes of airway mucus accumulation are largely speculative. There is evidence that mucus clearance is reduced in RAO. Findings in human airway diseases indicate that unfavorable alterations of mucus viscoelasticity can cause mucus stasis in the airways. Neutrophils, the predominant inflammatory cell type in RAO, can cause both unfavorable rheological alterations by neutrophil breakdown products like DNA and f-actin. Several authors have also suggested that mucus cell hyper- and metaplasia play a role in equine RAO, but this claim has so far not been substantiated by morphometric measurements. Since submucosal glands are sparse in horse airways, changes in epithelial goblet cells can be expected to account for most increased production and secretion of mucins in equine lower airway disease. Gel-forming mucins (MUC2, MUC5AC, MUC5B) have been

identified in several other species, and their expression and secretion is regulated within complex immunological networks. Neutrophil products as well as Th1 and Th2 cytokines that are involved in equine RAO can influence the expression of mucins, particularly of MUC5AC, in airway diseases of other species. Recent evidence from experimental models of airway disease further suggests that MUC5AC up-regulation converges on EGFR and CACC1 pathways in the airway epithelium.

Neither mucus accumulation nor mucus viscoelasticity nor mucin production and storage have been critically studied in equine RAO. The purpose of my work was therefore to quantify airway mucus accumulation and investigate its causes in RAO-affected horses and clinically healthy controls.

Hypotheses

1) Increased mucus accumulation is a function of disease status (RAO) and age of the horse as well as the environment. Further, increased mucus accumulation is associated with neutrophilic airway inflammation.

2) Increased mucus accumulation is a result of and associated with unfavorable alterations of mucus physical properties, i.e., increased viscoelasticity and decreased predicted clearability and/or

3) increased production of mucus, i.e., increased mucin gene mRNA levels and storage of mucosubstance in the airway epithelium.

Airway mucus accumulation

To develop the tool to test the first hypothesis, I validated an endoscopic scoring system. Endoscopic scoring has been previously used to assess changes in both mucus quantity and quality in horse airways. The validity of such scoring systems, however, has not been investigated. In order to determine if endoscopic scoring could be used to investigate airway mucus in horses, I investigated observer and horse variance of endoscopic scores and their relationship with measured variables.

Mucus accumulation scoring showed excellent interobserver agreement and moderate horse-related variance, was related to measured volumes of "artificial mucus", and correlated well with neutrophilic airway inflammation. Scores of mucus viscosity, color and localization, on the other hand, showed high observer-related variance, and did not correlate with measured mucus viscoelasticity. I concluded that apparent viscosity, localization and color scores should be interpreted with caution. In contrast, endoscopic scoring of mucus accumulation is a reliable clinical and research tool.

I then used endoscopic scoring of mucus accumulation to test the hypothesis that airway mucus accumulation is a function of disease status and/or environment. I compared mucus accumulation scores in RAO-affected and clinically healthy control horses before (when they were at pasture) and after (6, 24 and 48 hours) stabling on straw bedding and hay feeding.

I found that large amounts of mucus were specific for RAO-affected horses in this study. Variation among controls was considerable, however, and intermediate grades were non-specific, showing complete overlap between the two groups. During exacerbation, mucus accumulations increased 2-fold in RAO-affected horses, but

exposure to stable environment had no significant effect on mucus accumulations in controls, even though controls also showed a moderate increase of broncho-alveolar lavage fluid neutrophils. In conclusion, mucus accumulations, before and especially after exposure to dust and allergens, are increased in RAO-affected horses compared to controls. Healthy controls show considerable variability in mucus accumulation, but no increase of mucus accumulation after exposure to hay dust.

To investigate if airway mucus accumulation is also a function of age, I compared mucus accumulation scores in two age groups of clinically healthy well-performing sport horses that lived in the same controlled and constant stall environment. Several alternative outcomes were possible: no difference between age-groups; increased mucus in the older horses, as suggested by research in human smokers that produce more sputum with increasing pack-years; or, increased mucus in younger horses, as observed in racehorses entering training facilities.

I found that airway mucus accumulation did not differ between age groups. Clinically healthy older horses, having been exposed 10 more years to conventional stable environment without developing clinical signs of lower airway disease, do not show increased subclinical airway inflammation or mucus accumulation. Overall, however, large interindividual variations were observed in mucus quantity scores as well as broncho-alveolar lavage inflammatory cells. As previously observed in our research control horses, the majority of the asymptomatic well-performing sport horses were suffering from subclinical IAD. I propose that mild to moderate degrees of airway inflammation and mucus accumulation are a normal (i.e., observed in the majority of individuals) reaction to conventional stable environment, and that the significance of such

findings should be interpreted according to the level of respiratory performance expected from an individual.

The combination of these studies and reports on mucociliary clearance rates from other groups allowed for an estimate of the mucus volumes that are transported along the trachea in healthy and RAO-affected horses. I estimate that 2-8 ml per hour of endoscopically visible mucus are transported along the trachea in clinically healthy horses. In RAO-affected animals the volumes can be extrapolated to 6-14 ml per hour at pasture and 15-30 ml per hour when stabled. These estimates only account for mucus volumes cleared by mucociliary action, but not by coughing. A mean of 5 ml per hour corresponds to 120 ml/day for normal horses, while a mean of 22 ml/hr corresponds to 540 ml/day for the stabled RAO-affected animals. Given the size of a horse, the 120 ml/day normal value would be consistent with an estimate of about 10-15 ml/day in adult healthy humans, while the 540ml/day is proportionally less than the estimated 200-300 ml/day in humans with exacerbation of chronic bronchitis.

In contrast to the results in clinically healthy horses alone, mucus accumulation scores correlated well with tracheo-bronchial secretion neutrophil percentages and broncho-alveolar lavage fluid neutrophil numbers in horses that evenly represented the severity spectrum of non-infectious equine lower airway disease. Neutrophilic inflammation may cause mucus accumulation in horse airways through increased production and secretion of mucins and/or by unfavourably altering physical properties and clearability of the secretions.

Role of mucus rheology

I used the microrheometric method to test the second hypothesis that increased mucus accumulation is a result of and associated with unfavorable alterations of mucus physical properties, i.e., increased viscoelasticity and decreased predicted clearability. I compared mucus viscoelasticity and predicted clearability in RAO-affected and clinically healthy control horses before and after stabling on straw bedding and hay feeding.

The central findings were rheological changes in airway mucus, which occurred over time in RAO-affected animals, but not in controls. Mucus rheology was similar in both groups at baseline, but viscoelasticity increased about 3-fold on a linear scale in RAO-affected horses within 24 hours after environmental challenge, and was accompanied by significant decreases in predicted mucociliary and cough clearability. Rheological values did not correlate with the hydration of mucus or broncho-alveolar lavage fluid cytology. I concluded that viscoelastic properties of tracheal mucus samples from RAO-horses in remission do not differ from those of normal horses. However, environmental challenge causes acute exacerbation of airway obstruction and a concurrent increase in mucus viscoelasticity only in RAO-horses. Therefore, I inferred that unfavorable changes in mucus rheology may contribute to stasis and accumulation of mucus in RAO-horses in exacerbation, but not in clinical remission.

Although rheological values did not significantly correlate with broncho-alveolar lavage fluid cytology, the increase of mucus viscoelasticity coincided with the dramatic influx of neutrophils in the airways of RAO-affected horses. Neutrophils in the airways degenerate and release breakdown products, specifically DNA and filamentous (F)-actin that can unfavorably alter mucus rheological properties.

In order to test the hypothesis that neutrophil breakdown products cause unfavorable rheological changes, i.e., the observed increased mucus viscoelasticity during RAO exacerbation, I investigated the effects of DNase, which degrades DNA, and gelsolin, which degrades F-actin. I compared mucolytic effects on airway mucus from RAO-affected horses in exacerbation with effects on control mucus samples in a preliminary mucolytic study.

Gelsolin had a significant mucolytic effect on mucus of RAO horses in exacerbation, effectively reducing viscoelasticity almost 3-fold to normal levels, and indicating that F-actin plays a role in unfavorable rheological changes observed in mucus of RAO-affected horses during exacerbation. In contrast, DNase, at concentrations effective on cystic fibrosis sputum, did not alter viscoelasticity in RAO or control mucus. Thus, DNA fibers either do not play a similarly important rheological role in RAO mucus as in CF mucus, or, alternatively, they may be protected to a larger degree from mucolytic action. F-actin fibers have been shown to form an entangled network with DNA fibers, not only causing increases in viscoelasticity, but also protecting DNA fibers from mucolytic action by rhDNase. Further studies are needed to elucidate the role of DNA and F-actin fiber interactions in RAO mucus.

Role of mucin production

Unfavorable rheological properties leading to decreased mucus clearance can only partly explain the increased mucus accumulation in RAO, however. Particularly during remission, but likely also in exacerbation, increased production of mucus must also play a role. Based on findings in other species, any or all of the secreted, gel-forming mucins,

MUC2, MUC5AC and/or MUC5B, could be expressed in horse airways and may be involved in increased mucin production in RAO.

In order to test the hypothesis that increased production of mucus is a cause of the observed increased mucus accumulation, I identified equine homologues of gel-forming mucins and investigated the regional distribution of mRNA-levels of equine mucin gene homologues at different airway generations.

I identified equine (eq) homologues of MUC2 and MUC5AC, and found robust expression of eqMUC5AC in RAO and control horse airways, but any expression of MUC2 was undetectable and unlikely to be of physiological consequence. A semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay on pooled samples indicated increased levels of eqMUC5AC at large and small airway generations of RAO-affected horses. I consequently hypothesized that eqMUC5AC up-regulation may be a primary mechanism responsible for mucus hypersecretion and accumulation in RAO. MUC5AC is the main "signature" mucin in experimental and natural models of airway disease. Many different, Th-1 and -2 type as well as innate immunity, pathways converge on up-regulation of MUC5AC with subsequent mucous cell metaplasia and hypersecretion. Epidermal growth factor (EGF), its receptor, EGFR and homologues of the recently identified human calcium-activated chloride channel 1 (hCACC1) are key signaling molecules involved in these pathways, but have not been investigated in equine lung disease.

I therefore identified equine homologues of hCACC1 and EGFR and developed quantitative RT-PCR assays for eqMUC5AC, equine hCACC1-like, EGFR and EGF. This allowed me to accurately determine mRNA levels in individual samples and investigate the

association of eqMUC5AC expression with the expression of these key signaling elements, with stored intraepithelial mucosubstance and with neutrophilic inflammation. I studied samples from four lung lobes each in five RAO-affected and five clinically healthy control horses after the animals had been exposed to dusty stable environment for five days.

My findings did not support the hypothesis that eqMUC5AC is up-regulated and intraepithelial mucosubstances are increased in RAO-affected compared to clinically healthy control horses. Comparison with volume density of intraepithelial mucosubstance obtained by the same morphometric method in proximal and distal axial airways of rats, however, showed that means in horses of both disease groups were more than 10-fold higher than in untreated rats, and are in the range of values found in rats exposed to ozone and endotoxin. The relative, quantitative assessment of eqMUC5AC, equine hCACC1-like, EGFR or EGF mRNA levels showed remarkable consistency across different lung lobes within individuals, indicating that the underlying pathogenesis is a diffuse process involving the entire organ. Conversely, differences between horses were considerable. These differences reflect the variability in the degrees of mucus accumulation observed in RAO-affected and particularly also in clinically healthy horses. While the present results cannot explain the increased mucus in RAO airways, they may indicate that in some horses–bothRAO-affected and clinically healthy horses with milder degrees of airway disease–eqMUC5AC mRNA up-regulation may contribute to mucus hypersecretion. Furthermore, equine hCACC1-like and EGFR mRNA levels as well as neutrophil percentages were associated with eqMUC5AC mRNA levels in both groups. I therefore propose that these key signaling molecules play an important role in mucin regulation in

airways of horses suffering from RAO as well as of horses with milder degrees of airway inflammation.

Interpretation and outlook

Unfavourable rheological changes associated with F-actin can at least partly explain increased mucus accumulation during RAO exacerbation, but not in remission. An important unanswered question is whether therapeutic improvement of these unfavourable rheological changes is of any clinical benefit in RAO. It would be interesting to further study possible interactions and rheological effects of F-actin and DNA fibers. From a clinical perspective, however, peptide mucolytics such as gelsolin are at present cost prohibitive in the horse, and more practical solutions are needed. Since I have also shown good mucolytic *in vitro* efficacy of nacystelyn, it would be rational to test the clinical efficacy of an inexpensive equivalent of nacystelyn, such as N-acetyl-cysteine.

I could not support my hypothesis that increased mucin production, evidenced by eqMUC5AC expression, is a cause of the increased airway mucus in RAO. My results also cast doubt on the often cited subjective observations of mucus cell meta- and hyperplasia in RAO, since measurements of intraepithelial mucosubstance showed no difference between RAO-affected and control horses. It is important to note the high variability of mucus accumulation scores, neutrophils counts, stored intraepithelial mucosubstance and eqMUC5AC in RAO-affected horses as well as in control horses. Furthermore, compared with untreated laboratory rats, clinically healthy horses have highly increased mucus accumulation. Such cross-species comparisons can be

treacherous, however, and it would be a very worthwhile project to investigate the lungs and airways of horses that have never been exposed to indoor stable environments, for instance Mustangs.

Based on our present data, it seems that bronchospasm and not mucus accumulation and neutrophilic inflammation is the main defining pathophysiological characteristic of RAO. I propose that IAD, manifest with increased airway mucus and neutrophils, stored mucosubstances and mucin gene upregulation is very common in clinically healthy research horses. My results indicate that the clinical significance of these findings needs to be further determined with specific emphasis on the use of the animal, i.e., sport horse vs. racehorse.

Other areas that should be further explored are airway mucin biology and the causes of persistent mucus accumulation in RAO remission. In the former area, the equine homologue of MUC5B needs to be identified and mRNA levels of all gel-forming mucins must be assessed in combination with the identification and, if possible, quantification of their respective proteins products in airway secretions. The latter area is currently being addressed at the Pulmonary Laboratory. In addition to the investigation of the role of B-cell lymphoma 2 (Bcl-2) protein in the pathogenesis of persistent mucus accumulation in RAO remission, it would be very interesting to explore the link to the reported persistently increased levels of NF-κB and TNF-α. I plan to determine TNF-α, IFN-γ and, if possible, il-13 mRNA levels in the tissues from horses in acute exacerbation (chapter 8). Pending these results, the expression of this Th1 vs. Th2 cytokine balance may be particularly interesting in the context of resolution vs. persistence of mucus cell hyper- and metaplasia during RAO remission.

Two inherent limitations of the horse as a model for allergic lower airway disease have marked my thesis research: large interindividual variability due to underlying (subclinical) airway disease and difficulties of mechanistic interventions, i.e., lack of specific molecular blocking agents and in vitro systems. I use this as a legitimate excuse for the near absence of hard "mechanistic" facts, the prevalence of descriptive findings and the consequently mostly deductive reasoning that characterize my thesis research. None the wiser, I will continue to engage the challenges of "horse research".

I am intrigued by my observation that eqMUC5AC levels were associated with equine hCACC1-like, EGFR and neutrophils. Since the stable environment is similar for most of these horses, I propose that genetic differences in key signaling elements such as CACC1 and EGFR underlie the observed variability in clinical signs, airway mucus accumulation and mucin gene expression.

I plan to pursue this aspect. In the short term I will further investigate the genetic identity and single nucleotide polymorphisms of the equine hCACC1-like fragment, EGFR and later possibly eqMUC5AC and selected cytokines. I expect that any further investigations will have to contend with the noted interindividual variability in the horse. Two approaches are possible. Disease groups can be more rigorously defined so as to maximize differences between RAO-affected and control horses. Conversely, as a clinician, I plan to embrace the messy reality and investigate larger numbers of horses of varying degrees of non-infectious lower airway disease. My conceptual model is that of a continuous clinical spectrum due to overlapping, genetically determined gene expression profiles and resulting responses to environmental allergens and irritants. My long-term goal is to uncover some of the genetic background of the immunological and pathogenic

mechanisms that lead to non-infectious equine lower airway disease. At the University of Berne I will have access to a large number of horses of varying degrees of non-infectious lower airway disease to investigate the associations between clinical signs, pathophysiological abnormalities, gene expression data and genetic differences on a sound statistical basis. I also plan to explore the possibilities of using specific blockers, for instance for CACC1, EGFR and Th-2 type cytokines, in order to test more mechanistic hypotheses in the horse.

BIBLIOGRAPHY

1. Gerber, H., *Chronic pulmonary disease in the horse.* Equine Veterinary Journal, 1973. **5**: p. 26-33.

2. Dixon, P.M., *Respiratory mucociliary clearance in the horse in health and disease, and its pharmaceutical modification.* Vet Rec, 1992. **131**(11): p. 229-35.

3. Robinson, N.E., et al., *The pathogenesis of chronic obstructive pulmonary disease of horses.* Br Vet J, 1996. **152**(3): p. 283-306.

4. Matthews, L.M., et al., *Studies on pulmonary secretions.* Am. Rev. Respir. Dis., 1963. **88**: p. 199-204.

5. Dixon, P.M., D.I. Railton, and B.C. McGorum, *Equine pulmonary disease: a case control study of 300 referred cases. Part 2: details of animals and historical and clinical findings.* Equine Veterinary Journal, 1995. **27**: p. 422-427.

6. Woods, P.S., et al., *Airborne dust and aeroallergen concentration in a horse stable under two different management systems.* Equine Veterinary Journal, 1993. **25**(3): p. 208-13.

7. Pirie, R.S., *Role of inhaled endotoxin in the pathogenesis of equine COPD*, in *Proceedings of Workshop on Allergic Diseases of the Horse*, J.L. Watson, Editor. 1998: Lipica, Slovenia. p. 10.

8. McGorum, B.C., J. Ellison, and R.T. Cullen, *Total and respirable airborne dust endotoxin concentrations in three equine management systems.* Equine Veterinary Journal, 1998. **30**(5): p. 430-434.

9. Bracher, V., et al., *An investigation of the incidence of chronic obstructive pulmonary disease (COPD) in random populations of Swiss horses.* Equine Veterinary Journal, 1991. **23**: p. 136-141.

10. Sasse, H.H.L., S. Boerma, and F.A.A. Smolders, *The relationship between pulmonary function tests and other parameters. Results of a research project into the aetiology of COPD in horses*, in *Lung Function and Respiratory Diseases in the Horse*, R.E. Beadle, Editor. 1985, Hippiatrika: Hannover, Germany. p. 47-48.

11. Anon, *International Workshop on Equine Chronic Airway Disease. Proceedings of Workshop at Michigan State University, June 2000.* Eq. Vet. J., 2001. **33**: p. 5-19.

12. Anon, *International Workshop on Inflammatory Airway Disease: Defining the Syndrome. Proceedings of Workshop in Boston, October 2002.* Eq. Vet. Educ., 2002. **15**: p. 61-63.

13. McPherson, E.A., et al., *Chronic obstructive pulmonary disease (COPD): Identification of affected horses.* Equine Veterinary Journal, 1978. **10**: p. 47-53.

14. Fairbairn, S.M., et al., *Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure.* Clinical and Experimental Allergy, 1993. **23**: p. 821-828.

15. Derksen, F.J., et al., *Bronchoalveolar lavage in ponies with recurrent airway obstruction (heaves).* American Review of Respiratory Disease, 1985. **132**: p. 1066-1070.

16. Robinson, N.E., et al., *Determinants of maximal changes in pleural pressure in horses with heaves.* Proceedings of the 13th Veterinary Respiratory Symposium, 1994: p. A11.

17. Robinson, N.E., et al., *The airway response of horses with recurrent airway obstruction (heaves) to aerosol administration of ipratropium bromide.* Equine Veterinary Journal, 1993. **25**: p. 299-303.

18. Dixon, P.M., D.I. Railton, and B.C. McGorum, *Equine pulmonary disease: a case control study of 300 referred cases. Part 1: Examination techniques, diagnostic criteria and diagnoses.* Equine Veterinary Journal, 1995. **27**: p. 416-421.

19. Fairbairn, S.M., et al., *Duration of antigen-induced hyperresponsiveness in horses with allergic respiratory disease and possible links with early airway obstruction.* Journal of Veterinary Pharmacology and Therapeutics, 1993. **16**: p. 469-476.

20. Robinson, N.E., *Chairperson's introduction: International Workshop on Equine Chronic Airway Disease, Michigan State University, 16-18 June 2000.* Equine Veterinary Journal, 2001. **33**: p. 5-19.

21. Schatzmann, U., H. Buergi, and R. Straub, *Der Einfluß von Bisolvon auf das Tracheobronchialsekret des chronisch lungenkranken Pferdes [The influence of Bisolvon on tracheobronchial secretions of horses with chronic lung disease].* Deutsche Tierärztliche Wochenschrift, 1973. **80**: p. 97-100.

22. Gerber, V., et al., *Airway mucus and inflammation-short-term response to environmental challenge in clinically healthy control and in RAO-affected horses.* Journal of Veterinary Internal Medicine, 2003: p. submitted.

23. Gerber, H., *Nebenwirkungen von kortikosteroid-behandlungen beim grosstier.* Schweizer Archiv für Tierheilkunde, 1970. **112**: p. 51-57.

24. Robinson, N.E., et al., *Efficacy of three corticosteroids for the treatment of heaves.* Equine Veterinary Journal, 2002. **34**(1): p. 17-22.

25. Murphy, J.R., E.A. McPherson, and P.M. Dixon, *Chronic obstructive pulmonary disease (COPD): effects of bronchodilator drugs on normal and affected horses.* Equine Veterinary Journal, 1980. **12**(1): p. 10-4.

26. Soma, L.R., J. Beech, and N.H. Gerber, *Effects of cromolyn in horses with chronic obstructive pulmonary disease.* Veterinary Research Communications, 1987. **11**(4): p. 339-351.

27. Jackson, C.A., et al., *Environment and prednisone interactions in the treatment of recurrent airway obstruction (heaves).* Equine Veterinary Journal, 2000. **32**: p. 432-438.

28. Pearson, E.G., and T.W. Riebold, *Comparison of bronchodilators in alleviating clinical signs in horses with chronic obstructive pulmonary disease.* Journal of the American Veterinary Medical Association, 1989. **194**(9): p. 1287-91.

29. *International Workshop on Inflammatory Airway Disease: Defining the Syndrome. Proceedings of Workshop in Boston, October 2002.* . Eq. Vet. Edu., 2003. **in press.**

30. Hoffman, A.M., M.R. Mazan, and S. Ellenberg, *Association between bronchoalveolar lavage cytologic features and airway reactivity in horses with a history of exercise intolerance.* Am J Vet Res, 1998. **59**(2): p. 176-81.

31. Rose, R.J., et al., *Indices of exercise capacity in horses presented for poor performance.* Eq. Vet. J., 1995. **18**: p. 418-421.

32. Burrell, M.H., *Endoscopic and virological observations on respiratory disease in a group of young Thoroughbred horses in training.* Equine Veterinary Journal, 1985. **17**(2): p. 99-103.

33. MacNamara, B., S. Bauer, and J. Iafe, *Endoscopic evaluation of exercise-induced pulmonary hemorrhage and chronic obstructive pulmonary disease in association with poor performance in racing Standardbreds.* J Am Vet Med Assoc, 1990. **196**(3): p. 443-5.

34. Sweeney, C.R., K.A. Humber, and K.A. Roby, *Cytologic findings of tracheobronchial aspirates from 66 thoroughbred racehorses.* Am J Vet Res, 1992. **53**(7): p. 1172-5.

35. Martin, B.B., Jr., J. Beech, and E.J. Parente, *Cytologic examination of specimens obtained by means of tracheal washes performed before and after high-speed treadmill exercise in horses with a history of poor performance.* Journal of the American Veterinary Medical Association, 1999. **214**(5): p. 673-677.

36. Morris, E.A. and H.J. Seeherman, *Clinical evaluation of poor performance in the racehorse: the results of 275 evaluations.* Equine Vet J, 1991. **23**(3): p. 169-74.

37. Martin, B.B., Jr., et al., *Causes of poor performance of horses during training, racing, or showing: 348 cases (1992-1996).* J Am Vet Med Assoc, 2000. **216**(4): p. 554-8.

38. Moreno, R.H., J.C. Hogg, and P.D. Pare, *Mechanics of airway narrowing.* American Review of Respiratory Disease, 1986. **133**: p. 1171-1180.

39. Kim, W.D., *Lung mucus: a clinician's view.* European Respiratory Journal, 1997. **10**: p. 1914-1917.

40. Sherman, C.B., et al., *Longitudinal lung function decline in subjects with respiratory symptoms.* Am Rev Respir Dis, 1992. **146**(4): p. 855-9.

41. Annesi, I. and F. Kauffmann, *Is respiratory mucus hypersecretion really an innocent disorder? A 22-year mortality survey of 1,061 working men.* Am Rev Respir Dis, 1986. **134**(4): p. 688-93.

42. Broadstone, R.V., et al., *Effects of atropine in ponies with recurrent airway obstruction.* Journal of Applied Physiology, 1988. **65**: p. 2720-2725.

43. Derksen, F.J., N.E. Robinson, and C.E. Berney, *Aerosol pirbuterol: bronchodilator activity and side effects in ponies with recurrent airway obstruction (heaves).* Equine Veterinary Journal, 1992. **24**: p. 107-112.

44. Rush, B.R., et al., *Pulmonary distribution of aerosolized technetium Tc 99m pentetate after administration of a single dose of aerosolized albuterol sulfate in horses with recurrent airway obstruction.* American Journal of Veterinary Research, 1999. **60**: p. 764-769.

45. Schatzmann, U., R. Straub, and H. Gerber, *[Bronchial secretion aspiration in the horse].* Schweiz Arch Tierheilkd, 1972. **114**(8): p. 395-403.

46. Dieckmann, M.P., *Zur Wirksamkeit von Ambroxolhydrochlorid (Mukovent) bei lungenkranken Pferden - klinische, Funktionelle und zytologische Untersuchungen*, in *Tieraerztliche Hochschule.* 1987: Hannover, Germany.

47. Holcombe, S.J., et al., *Stabling is associated with airway inflammation in young Arabian horses.* Equine Vet J, 2001. **33**(3): p. 244-9.

48. Luft, J., *Der Einfluss koerperlicher Belastung auf die Zytologie des Tracheobronchialsekretes beim Pferd [the effect of exercise of the composition of tracheobronchial secretions in the horse].* Dissertation, 1987. **Tieraerztliche Hochschule Hannover, Germany**.

49. Dixon, P.M., D.I. Railton, and B.C. McGorum, *Equine pulmonary disease: a case control study of 300 referred cases. Part 3: Ancillary diagnostic findings.* Equine Veterinary Journal, 1995. **27**: p. 428-435.

50. Christley, R.M., et al., *Coughing in thoroughbred racehorses: risk factors and tracheal endoscopic and cytological findings.* Vet Rec, 2001. **148**(4): p. 99-104.

51. Robinson, N.E., et al., *Cough, mucus, airway obstruction, and inflammation in heaves-affected and control horses.* American Journal of Veterinary Research, 2003. **64**(5): p. 550-557.

52. Raidal, S.L., D.N. Love, and G.D. Bailey, *Inflammation and increased numbers of bacteria in the lower respiratory tract of horses within 6 to 12 hours of confinement with the head elevated.* Aust Vet J, 1995. **72**(2): p. 45-50.

53. Chapman, P.S., et al., *Retrospective study of the relationships between age, inflammation and the isolation of bacteria from the lower respiratory tract of thoroughbred horses.* Vet Rec, 2000. **146**(4): p. 91-5.

54. Jefcoat, A.M., et al., *Persistent mucin glycoprotein alterations in equine recurrent airway obstruction.* Am J Physiol Lung Cell Mol Physiol, 2001. **281**(3): p. L704-12.

55. Sweeney, C.R., J. Beech, and K.A. Roby, *Bacterial isolates from tracheobronchial aspirates of healthy horses.* Am J Vet Res, 1985. **46**(12): p. 2562-5.

56. Houtmeyers, E., et al., *Regulation of mucociliary clearance in health and disease.* Eur Respir J, 1999. **13**(5): p. 1177-88.

57. Maxson, A.D., et al., *Effects of furosemide, exercise, and atropine on tracheal mucus transport rate in horses.* Am J Vet Res, 1995. **56**(7): p. 908-12.

58. Turgut, K. and H.H. Sasse, *Influence of clenbuterol on mucociliary transport in healthy horses and horses with chronic obstructive pulmonary disease.* Vet Rec, 1989. **125**(21): p. 526-30.

59. Sweeney, C.R., et al., *Effect of water vapor-saturated air therapy on bronchoalveolar lavage and tracheal mucus transport rate in clinically normal horses.* Am J Vet Res, 1989. **50**(2): p. 276-9.

60. Raidal, S.L., *The incidence and consequences of failure of passive transfer of immunity on a thoroughbred breeding farm.* Aust Vet J, 1996. **73**(6): p. 201-6.

61. Gerber, V., et al., *Influence of gravity on tracheal mucus velocity in excised horse tracheas.* J Respir Crit Care Med, 1996. **153**(4): p. A483.

62. Schlesinger, R.B., *Clearance from the respiratory tract.* Fund. and Appl. Tox., 1985. **5**: p. 435-50.

63. Del Donno, M., et al., *The effect of inflammation on mucociliary clearance in asthma: an overview.* Chest, 2000. **118**(4): p. 1142-9.

64. Coombs, S.L. and P.M. Webbon, *Observations on tracheal mucociliary clearance in horses.* Tierarztl Prax Suppl, 1987. **2**: p. 5-9.

65. Willoughby, R.A., et al., *Use of scintigraphy for the determination of mucociliary clearance rates in normal, sedated, diseased and exercised horses.* Canadian Journal of Veterinary Research, 1991. **55**: p. 315-320.

66. Willoughby, R., et al., *The effects of equine rhinovirus, influenza virus and herpesvirus infection on tracheal clearance rate in horses.* Can J Vet Res, 1992. **56**(2): p. 115-21.

67. Sweeney, C.R., *Tracheal mucus transport rate in healthy horses.* Am J Vet Res, 1989. **50**(12): p. 2135-7.

68. Camner, P., K. Philipson, and L. Friberg, *Tracheobronchial clearance in twins.* Arch. Environ. Health, 1971. **23**: p. 82-7.

69. King, M. and B.K. Rubin, *Rheology of airway mucus: relationship with clearance function*, in *Airway Secretion: Physiological Bases for the Control of Mucous Hypersecretion*, S. Shimura, Editor. 1994, Marcel Dekker, Inc.: New York. p. 283-314.

70. Kaup, F.J., W. Drommer, and E. Deegen, *Ultrastructural findings in horses with chronic obstructive pulmonary disease (COPD). I: Alterations of the larger conducting airways.* Equine Vet J, 1990. **22**(5): p. 343-8.

71. Galati, P., et al., *Atypical cilia in the tracheal epithelium of healthy horses.* J Comp Pathol, 1991. **105**(2): p. 185-90.

72. Gerber, V., et al., *Microscopic investigation of mucus qualities, mucus transport velocity and ciliary beat frequency on horse tracheal epithelium.* Respiration Physiology, 1997. **107**: p. 67-74.

73. Jeanneret-Grosjean, A., et al., *Sampling technique and rheology of human tracheobronchial mucus.* Am Rev Respir Dis, 1988. **137**(3): p. 707-10.

74. Girod, S., et al., *Phospholipid composition and surface-active properties of tracheobronchial secretions from patients with cystic fibrosis and chronic obstructive pulmonary diseases.* Pediatr Pulmonol, 1992. **13**(1): p. 22-7.

75. Gerber, V., *Untersuchungen über die mukoziliäre Clearance in der Luftröhre des Pferdes [Investigation of mucociliary clearance in equine trachea].* Dissertation, 1995. **University of Berne, Switzerland**.

76. Im Hof, V., et al., *Exogenous surfactant accelerates tracheal mucus velocity in spontanously breathing non-anaesthetized horses.* J Respir Crit Care Med, 1996. **153**(4): p. A109.

77. Im Hof, V., et al., *In vivo determination of surface tension in the horse trachea and in vitro model studies.* Respir Physiol, 1997. **109**(1): p. 81-93.

78. Dorwald, M., G. Vanden Bosche, and A. Gerull, *Zur Surfactantphospholipidzusammensetzung im Tracheobronchialsekret des Pferdes und ihrer klinischen relevanz fuer die Beurteilung des Lungenstatus bei chronisch lungenkranken Pferden.* Wien Tieraerztl. Mschr., 1991. **78**: p. 118-26.

79. Hobo, S., et al., *Surfactant proteins in bronchoalveolar lavage fluid of horses: assay technique and changes following road transport.* Vet Rec, 2001. **148**(3): p. 74-80.

80. Boat, T.F., R.-W. Cheng, and M. Warren Leigh, *Biochemistry of mucus*, in *Airway secretion: Physiological bases for the control of mucous hypersecretion*, S. Shimura, Editor. 1995, Marcel Dekker, Inc.: New York. p. 217-82.

81. McGorum, B.C., et al., *Evaluation of urea and albumen as endogenous markers of dilution of equine bronchoalveolar lavage fluid.* Res Vet Sci, 1993. **55**(1): p. 52-6.

82. Marcy, T.W., et al., *Limitations of using urea to quantify epithelial lining fluid recovered by bronchoalveolar lavage.* Am Rev Respir Dis, 1987. **135**(6): p. 1276-80.

83. Persson, C.G., *Airway Mucosal Exudation of Plasma*, in *Airway secretion: Physiological bases for the control of mucous hypersecretion*, S. Shimura, Editor. 1995, Marcel Dekker, Inc.: New York. p. 451-67.

84. Winder, N.C. and R. von Fellenberg, *Immunofluorescent evaluation of the lower respiratory tract of healthy horses and of horses with chronic bronchiolitis.* American Journal of Veterinary Research, 1986. **47**: p. 1271-1274.

85. Rose, M.C., *Mucins: structure, function, and role in pulmonary diseases.* American Journal of Physiology: Lung Cellular and Molecular Physiology, 1992. **263**: p. L413-L429.

86. Gendler, S.J. and A.P. Spicer, *Epithelial mucin genes.* Annual Review of Physiology, 1995. **57**: p. 607-634.

87. Jeffery, P.K. and D. Li, *Airway mucosa: secretory cells, mucus, and mucin genes.* European Respiratory Journal, 1997. **10**: p. 1655-1662.

88. Perez-Vilar, J., J.K. Sheehan, and S.H. Randell, *Making More MUCS.* Am J Respir Cell Mol Biol, 2003. **28**(3): p. 267-70.

89. Lundgren, J.D. and J.H. Shelhamer, *Pathogenesis of airway mucus hypersecretion.* J Allergy Clin Immunol, 1990. **85**(2): p. 399-417.

90. Thornton, D.J., et al., *Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule.* J Biol Chem, 1997. **272**(14): p. 9561-6.

91. Kirkham, S., et al., *Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B.* Biochem J, 2002. **361**(Pt 3): p. 537-46.

92. Thornton, D.J., et al., *Respiratory mucins: identification of core proteins and glycoforms.* Biochem J, 1996. **316 (Pt 3)**: p. 967-75.

93. Leikauf, G.D., et al., *Mucin apoprotein expression in COPD.* Chest, 2002. **121**(5 Suppl): p. 166S-182S.

94. Chen, Y., Y.H. Zhao, and R. Wu, *In silico cloning of mouse Muc5b gene and upregulation of its expression in mouse asthma model.* Am J Respir Crit Care Med, 2001. **164**(6): p. 1059-66.

95. Borchers, M.T., M.P. Carty, and G.D. Leikauf, *Regulation of human airway mucins by acrolein and inflammatory mediators.* Am J Physiol, 1999. **276**(4 Pt 1): p. L549-55.

96. Basbaum, C., et al., *Control of mucin transcription by diverse injury-induced signaling pathways.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 2): p. S44-8.

97. Harkema, J.R. and J.G. Wagner, *Non-allergic models of mucous cell metaplasia and mucus hypersecretion in rat nasal and pulmonary airways.* Novartis Found Symp, 2002. **248**: p. 181-97; discussion 197-200, 277-82.

98. Jany, B., [*Molecular cloning of human respiratory tract mucin and studies of mucin gene expression in tracheobronchial epithelium of the rat].* Pneumologie, 1993. **47**(8): p. 479-87.

99. Li, D., et al., *Localization and up-regulation of mucin (MUC2) gene expression in human nasal biopsies of patients with cystic fibrosis.* J Pathol, 1997. **181**(3): p. 305-10.

100. Bullimore, S.R., et al., *Surface mucus in the non-glandular region of the equine stomach.* Res Vet Sci, 2001. **70**(2): p. 149-55.

101. Kemmish, A.R., et al. *Biochemical comparison of respiratory mucins from normal horses and horses with chronic inflammatory small airway disease.* in *British Equine Veterinary Association*. 1998. Birmingham.

102. Walley, E.A., et al. *Characterization of equine respiratory tract mucins.* in *World Equine Airway Symposium*. 2001. Edinburgh.

103. Scharfman, A., et al., *Recognition of Lewis x derivatives present on mucins by flagellar components of Pseudomonas aeruginosa.* Infect Immun, 2001. **69**(9): p. 5243-8.

104. Trivier, D., et al., *The binding of surface proteins from Staphylococcus aureus to human bronchial mucins.* Eur Respir J, 1997. **10**(4): p. 804-10.

105. Majima, Y., et al., *Effect of biochemical components on rheologic properties of nasal mucus in chronic sinusitis.* Am J Respir Crit Care Med, 1999. **160**(2): p. 421-6.

106. Kurashima, K., et al., *Effect of thromboxane A2 synthetase inhibitor, OKY-046, on sputum in chronic bronchitis and diffuse panbronchiolitis.* Eur Respir J, 1995. **8**(10): p. 1705-11.

107. Groneberg, D.A., et al., *Distribution of respiratory mucin proteins in human nasal mucosa.* Laryngoscope, 2003. **113**(3): p. 520-4.

108. Widdicombe, J.H. and I.S. Pecson, *Distribution and numbers of mucous glands in the horse trachea.* Equine Vet J, 2002. **34**(6): p. 630-3.

109. Reid, L.M., *Pathology of chronic bronchitis.* Lancet, 1954. **1**: p. 275-276.

110. Dunnill, M.D., M. G.R., and J.A. Anderson, *A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema.* Thorax, 1969. **24**: p. 176-179.

111. Aikawa, T., et al., *Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack.* Chest, 1992. **101**: p. 916-921.

112. Hotchkiss, J.A., Guelph, Canada. *Airway mucous synthesis, storage, and secretion.* in *World Equine Airways Symposium*. 1998. Guelph, Canada.

113. Haschek, W.M., *Respiratory system*, in *Toxicologic Pathology*, C.G. Rousseaux, Editor. 1998, Academic Press: San Diego. p. 108-22.

114. Strieter, R.M., J.A. Belperio, and M.P. Keane, *Cytokines in innate host defense in the lung.* J Clin Invest, 2002. **109**(6): p. 699-705.

115. Wills-Karp, M., *IL-12/IL-13 axis in allergic asthma.* J Allergy Clin Immunol, 2001. **107**(1): p. 9-18.

116. Romagnani, S., *The Th1/Th2 paradigm.* Immunol Today, 1997. **18**(6): p. 263-6.

117. Holgate, S.T., *The immunopharmacology of mild asthma.* J Allergy Clin Immunol, 1996. **98**(5 Pt 2): p. S7-16; discussion S33-40.

118. Longphre, M., et al., *Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells.* J Clin Invest, 1999. **104**(10): p. 1375-82.

119. Soussi-Gounni, A., M. Kontolemos, and Q. Hamid, *Role of IL-9 in the pathophysiology of allergic diseases.* J Allergy Clin Immunol, 2001. **107**(4): p. 575-82.

120. Grunig, G., et al., *Roles of interleukin-13 and interferon-gamma in lung inflammation.* Chest, 2002. **121**(3 Suppl): p. 88S.

121. Morokata, T., J. Ishikawa, and T. Yamada, *Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses.* Immunol Lett, 2000. **72**(2): p. 119-26.

122. Dearman, R.J. and I. Kimber, *Cytokine profiling and chemical allergy.* Toxicol Appl Pharmacol, 2002. **185**(3): p. 228-9; author reply 229-30.

123. Dearman, R.J. and I. Kimber, *Cytokine fingerprinting: characterization of chemical allergens.* Methods, 1999. **19**(1): p. 56-63.

124. Tulic, M.K., P.G. Holt, and P.D. Sly, *Modification of acute and late-phase allergic responses to ovalbumin with lipopolysaccharide.* Int Arch Allergy Immunol, 2002. **129**(2): p. 119-28.

125. Anderson, G.P., *The immunobiology of early asthma.* Med J Aust, 2002. **177 Suppl**: p. S47-9.

126. Jeffery, P.K., *Lymphocytes, chronic bronchitis and chronic obstructive pulmonary disease.* Novartis Found Symp, 2001. **234**: p. 149-61; discussion 161-8.

127. Wills-Karp, M. and M. Chiaramonte, *Interleukin-13 in asthma.* Curr Opin Pulm Med, 2003. **9**(1): p. 21-7.

128. Riffo-Vasquez, Y. and D. Spina, *Role of cytokines and chemokines in bronchial hyperresponsiveness and airway inflammation.* Pharmacol Ther, 2002. **94**(3): p. 185-211.

129. McMillan, S.J., et al., *The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity.* J Exp Med, 2002. **195**(1): p. 51-7.

130. Grunig, G., et al., *Requirement for IL-13 independently of IL-4 in experimental asthma.* Science, 1998. **282**(5397): p. 2261-3.

131. Nakanishi, A., et al., *Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5175-80.

132. Toda, M., et al., *A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma.* J Allergy Clin Immunol, 2002. **109**(2): p. 246-50.

133. Zhou, Y., et al., *Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma.* Am J Respir Cell Mol Biol, 2001. **25**(4): p. 486-91.

134. Shim, J.J., et al., *IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(1): p. L134-40.

135. Takeyama, K., et al., *Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils.* Journal of Immunology, 2000. **164**(3): p. 1546-52.

136. Fahy, J.V., *Goblet cell and mucin gene abnormalities in asthma.* Chest, 2002. **122**(6 Suppl): p. 320S-326S.

137. Becker, S., M.J. Fenton, and J.M. Soukup, *Involvement of microbial components and toll-like receptors 2 and 4 in cytokine responses to air pollution particles.* Am J Respir Cell Mol Biol, 2002. **27**(5): p. 611-8.

138. Wang, X., et al., *Toll-like receptor 4 mediates innate immune responses to Haemophilus influenzae infection in mouse lung.* J Immunol, 2002. **168**(2): p. 810-5.

139. Shi, Z.O., et al., *IFN-gamma, but not Fas, mediates reduction of allergen-induced mucous cell metaplasia by inducing apoptosis.* J Immunol, 2002. **168**(9): p. 4764-71.

140. Tesfaigzi, Y., et al., *Bcl-2 in LPS- and allergen-induced hyperplastic mucous cells in airway epithelia of Brown Norway rats.* American Journal of Physiology: Lung Cellular and Molecular Physiology, 2000. **279**(6): p. L1210-7.

141. Pack, R.J., L.H. Al-Ugaily, and G. Morris, *The cells of the tracheobronchial epithelium of the mouse: a quantitative light and electron microscope study.* J Anat, 1981. **132**(Pt 1): p. 71-84.

142. Mercer, R., et al., *Cell number and distribution in human and rat airways.* Am. J. Respir. Cell Mol. Biol., 1994. **10**(6): p. 613-624.

143. Zhu, Z., et al., *Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.* J. Clin. Invest., 1999. **103**(6): p. 779-788.

144. Temann, U.A., et al., *A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion.* Am J Respir Cell Mol Biol, 1997. **16**(4): p. 471-8.

145. Kim, C.H., et al., *IL-13 suppresses MUC5AC gene expression and mucin secretion in nasal epithelial cells.* Acta Otolaryngol, 2002. **122**(6): p. 638-43.

146. Seong, J.K., et al., *Upregulation of MUC8 and downregulation of MUC5AC by inflammatory mediators in human nasal polyps and cultured nasal epithelium.* Acta Otolaryngol, 2002. **122**(4): p. 401-7.

147. Jayawickreme, S.P., et al., *Regulation of 15-lipoxygenase expression and mucus secretion by IL-4 in human bronchial epithelial cells.* Am J Physiol, 1999. **276**(4 Pt 1): p. L596-603.

148. Sonea, I.M., R.M. Bowker, and N.E. Robinson, *Distribution of substance P binding sites in equine airways.* Equine Vet J, 1999. **31**(3): p. 238-42.

149. Olszewski, M.A., et al., *Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways.* American Journal of Physiology, 1999. **276**: p. L522-29.

150. Takeyama, K., et al., *Neutrophil-dependent goblet cell degranulation: role of membrane-bound elastase and adhesion molecules.* American Journal of Physiology: Lung Cellular and Molecular Physiology, 1998. **275**(2 Pt 1): p. L294-302.

151. Takeyama, K., et al., *Epidermal growth factor system regulates mucin production in airways.* Proceedings of the National Academy of Sciences, USA, 1999. **96**(6): p. 3081-6.

152. Art, T., et al., *Indices of oxidative stress in blood and pulmonary epithelium lining fluid in horses suffering from recurrent airway obstruction.* Equine Veterinary Journal, 1999. **31**: p. 397-401.

153. Lindberg, A., et al., *Evaluation of leukotriene biosynthetic capacity in lung tissues from horses with recurrent airway obstruction.* Am J Vet Res, 2002. **63**(6): p. 794-8.

154. Halliwell, R.E., et al., *Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease.* Vet Immunol Immunopathol, 1993. **38**(3-4): p. 201-15.

155. Ainsworth, D.M., et al., *IgG antibody responses to an inhaled antigen in horses with "heaves" (recurrent airway obstruction).* Vet Immunol Immunopathol, 2002. **84**(3-4): p. 169-80.

156. Pirie, R.S., P.M. Dixon, and B.C. McGorum, *Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3: Effect of fractionation of HDS.* Equine Vet J, 2002. **34**(4): p. 343-7.

157. Pirie, R.S., et al., *Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses.* Equine Vet J, 2002. **34**(4): p. 337-42.

158. Lavoie, J.P., et al., *Neutrophilic Airway Inflammation in Horses with Heaves Is Characterized by a Th2-type Cytokine Profile.* American Journal of Respiratory Critical Care Medicine, 2001. **164**(8): p. 1410-3.

159. Beadle, R.E., D.W. Horohov, and S.D. Gaunt, *Interleukin-4 and interferon-gamma gene expression in summer pasture-associated obstructive pulmonary disease affected horses.* Equine Vet J, 2002. **34**(4): p. 389-94.

160. Bowles, K.S., et al., *A novel model for equine recurrent airway obstruction.* Vet Immunol Immunopathol, 2002. **87**(3-4): p. 385-9.

161. Giguere, S., et al., *Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate.* Vet Immunol Immunopathol, 2002. **85**(3-4): p. 147-58.

162. Fanucchi, M.V., J.A. Hotchkiss, and J.R. Harkema, *Endotoxin potentiates ozone-induced mucous cell metaplasia in rat nasal epithelium.* Toxicol Appl Pharmacol, 1998. **152**(1): p. 1-9.

163. Gordon, T., et al., *One-month exposure to inhaled endotoxin produces a dose-dependent increase in stored mucosubstances in rat intrapulmonary airways.* Exp Lung Res, 1996. **22**(5): p. 509-23.

164. Bureau, F., et al., *Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma.* American Journal of Respiratory Critical Care Medicine, 2000. **161**(4 Pt 1): p. 1314-21.

165. Bureau, F., et al., *An autocrine mechanism maintains persistent NF-B activation in the bronchi of an animal model of asthma.* American Journal of Respiratory Critical Care Medicine, 2000. **161**: p. A330.

166. Koo, J.S., et al., *Overexpression of mucin genes induced by interleukin-1 beta, tumor necrosis factor-alpha, lipopolysaccharide, and neutrophil elastase is inhibited by a retinoic acid receptor alpha antagonist.* Exp Lung Res, 2002. **28**(4): p. 315-32.

167. Herholz, C., et al., *Relationship between clinical signs and pulmonary function estimated by the single breath diagram for CO(2) (SBD-CO(2)) in horses with chronic obstructive pulmonary disease.* Vet J, 2002. **163**(2): p. 187-95.

168. King, M., *Magnetic microrheometer*, in *Methods in Bronchial Mucology*, L. Allegra, Editor. 1988, Raven Press, Ltd.: New York. p. 73-83.

169. Gerber, V., et al., *Tracheobronchial mucus viscoelasticity during environmental challenge in horses with recurrent airway obstruction.* Equine Vet J, 2000. **32**(5): p. 411-7.

170. Fischer, B. and J. Voynow, *Neutrophil elastase induces MUC5AC messenger RNA expression by an oxidant-dependent mechanism.* Chest, 2000. **117**(5 Suppl 1): p. 317S-20S.

171. Armstrong, J. and J. White, *Liquefaction of viscous purulent exudates by deoxyribonuclease.* Lancet, 1950. **2**: p. 739-742.

172. Pietra, M., et al., *In vitro mucolytic activity of recombinant human deoxyribonuclease on equine tracheobronchial mucus.* Vet Rec, 2000. **147**(22): p. 627-9.

173. Keller, H., et al., *Klinische Studie zur Wirksamkeit und Verträglichkeit von N-Acetylcystein bei der Behandlung der COB/COPD des Pferdes [efficacy and tolerance study of acetylcysteine in the COB/COPD therapy in horses].* Prakt. Tierarzt, 2001. **82**: p. 108-117.

174. Tremblay, G.M., et al., *Effect of stabling on bronchoalveolar cells obtained from normal and COPD horses.* Equine Vet J, 1993. **25**(3): p. 194-7.

175. Voynow, J.A., et al., *Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells.* American Journal of Physiology, 1999. **276**(5 Pt 1): p. L835-43.

176. Wagner, J.G., et al., *Endotoxin enhancement of ozone-induced mucous cell metaplasia is neutrophil-dependent in rat nasal epithelium.* Toxicol Sci, 2001. **60**(2): p. 338-47.

177. Rubin, B.K., *The pharmacologic approach to airway clearance: mucoactive agents.* Respir Care, 2002. **47**(7): p. 818-22.

178. Robinson, N.E., et al., *Relationship between clinical signs and lung function in horses with recurrent airway obstruction (heaves) during a bronchodilator trial.* Equine Veterinary Journal, 2000. **32**: p. 393-400.

179. Gerber, V., et al., *Endoscopic scoring of mucus: Reproducibility, and relation to mucus viscosity and airway inflammation.* EVJ, 2003. **in preparation.**

180. Tremblay, G.M., et al., *Effect of stabling on bronchoalveolar cells obtained from normal and COPD horses.* Equine Veterinary Journal, 1993. **25**(3): p. 194-197.

181. Bracher, V., et al., *An investigation of the incidence of chronic obstructive pulmonary disease (COPD) in random populations of Swiss horses.* Equine Vet J, 1991. **23**(2): p. 136-41.

182. Gerber, V., et al., *Airway mucus in RAO - short-term response to environmental challenge.* Journal of Veterinary Internal Medicine, 2003: p. accepted.

183. Zayas, J.G., G.C. Man, and M. King, *Tracheal mucus rheology in patients undergoing diagnostic bronchoscopy. Interrelations with smoking and cancer.* Am Rev Respir Dis, 1990. **141**(5 Pt 1): p. 1107-13.

184. Viel, L. and J. Hewson, *BAL cytology in horses with exercise intolerance: what does it tell us?* Second World Equine Airways Symposium (WEAS) proceedings, Edinburgh, Scotland., 2001.

185. Sutton, G.A., et al., *Pathogenesis and clinical signs of equine herpesvirus-1 in experimentally infected ponies in vivo.* Can J Vet Res, 1998. **62**(1): p. 49-55.

186. Derksen, F.J., et al., *Comparison of transtracheal aspirate and bronchoalveolar lavage cytology in 50 horses with chronic lung disease.* Equine Veterinary Journal, 1989. **21**: p. 23-26.

187. Hoffman, A. *Airway reactivity in the assessment of lung function.* in *Second World Equine Airways Symposium (WEAS).* 2001. Edinburgh, Scottland: WEAS.

188. Gerber, V., et al., *Mucus quality on horse tracheal epithelium: microscopic grading based on transparency.* Respir Physiol, 1997. **107**(1): p. 67-74.

189. App, E.M., J.G. Zayas, and M. King, *Rheology of mucus and transepithelial potential difference: small airways versus trachea.* Eur Respir J, 1993. **6**(1): p. 67-75.

190. Shih, C.K., et al., *Effect of nondialyzable solids concentration and viscoelasticity on ciliary transport of tracheal mucus.* American Review of Respiratory Disease, 1977. **115**: p. 989-995.

191. Picot, R., I. Das, and L. Reid, *Pus, deoxyribonucleic acid, and sputum viscosity.* Thorax, 1978. **33**(2): p. 235-42.

192. Vasconcellos, C., et al., *Reduction in viscosity of cystic fibrosis sputum in vitro by gelsolin.* Science, 1994. **263**: p. 969-971.

193. Tomkiewicz, R.P., et al., *Species differences in the physical and transport properties of airway secretions.* Can J Physiol Pharmacol, 1995. **73**(2): p. 165-71.

194. Dasgupta, B., N.E. Brown, and M. King, *Effects of sputum oscillations and rhDNase in vitro: a combined approach to treat cystic fibrosis lung disease.* Pediatr Pulmonol, 1998. **26**(4): p. 250-5.

195. Gerber, V., et al. *Rheology of mucus in horses with moderate COPD.* in *World Equine Airways Symposium.* 1998. Guelph, Canada: WEAS.

196. King, M., *Relationship between mucus viscoelasticity and ciliary transport in guaran gel/frog palate model system.* Biorheology, 1980. **17**(3): p. 249-54.

197. King, M., *Role of mucus viscoelasticity in clearance by cough.* Eur J Respir Dis Suppl, 1987. **153**: p. 165-72.

198. King, M., M. Agarwal, and J.B. Shukla, *A planar model for mucociliary transport: effect of mucus viscoelasticity.* Biorheology, 1993. **30**(1): p. 49-61.

199. Dasgupta, B., et al., *Rheological properties in cystic fibrosis airway secretions with combined rhDNase and gelsolin treatment,* in *Advances in Physiological Fluid Dynamics*, V.P. Saxena, Editor. 1996, Narosa: New Delhi, India. p. 74-78.

200. Dasgupta, B. and M. King, *Reduction in viscoelasticity in cystic fibrosis sputum in vitro using combined treatment with nacystelyn and rhDNase.* Pediatr Pulmonol, 1996. **22**(3): p. 161-6.

201. Sheils, C.A., et al., *Actin filaments mediate DNA fiber formation in chronic inflammatory airway disease.* Am J Pathol, 1996. **148**(3): p. 919-27.

202. Zahm, J.M., et al., *Improved activity of an actin-resistant DNase I variant on the cystic fibrosis airway secretions.* Am J Respir Crit Care Med, 2001. **163**(5): p. 1153-7.

203. Buisine, M.P., et al., *Developmental mucin gene expression in the human respiratory tract.* Am J Respir Cell Mol Biol, 1999. **20**(2): p. 209-18.

204. Davies, J.R., et al., *Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions.* Biochem J, 1999. **344 Pt 2**: p. 321-30.

205. Groneberg, D.A., et al., *Expression of respiratory mucins in fatal status asthmaticus and mild asthma.* Histopathology, 2002. **40**(4): p. 367-73.

206. Thornton, D.J., et al., *Characterization of mucins from cultured normal human tracheobronchial epithelial cells.* Am J Physiol Lung Cell Mol Physiol, 2000. **278**(6): p. L1118-28.

207. Voynow, J.A., *What does mucin have to do with lung disease?* Paediatr Respir Rev, 2002. **3**(2): p. 98-103.

208. Chomczynski, P. and N. Sacchi, *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Anal Biochem, 1987. **162**(1): p. 156-9.

209. Guzman, K., T. Bader, and P. Nettesheim, *Regulation of MUC5 and MUC1 gene expression: correlation with airway mucous differentiation.* Am J Physiol, 1996. **270**(5 Pt 1): p. L846-53.

210. Steiger, D., et al., *Use of mucin antibodies and cDNA probes to quantify hypersecretion in vivo in human airways.* Am J Respir Cell Mol Biol, 1994. **10**(5): p. 538-45.

211. Perez-Vilar, J. and R.L. Hill, *Identification of the half-cystine residues in porcine submaxillary mucin critical for multimerization through the D-domains. Roles of the CGLCG motif in the D1- and D3-domains.* J Biol Chem, 1998. **273**(51): p. 34527-34.

212. Van Klinken, B.J., et al., *Mucin gene structure and expression: protection vs. adhesion.* Am J Physiol, 1995. **269**(5 Pt 1): p. G613-27.

213. Louahed, J., et al., *Interleukin-9 upregulates mucus expression in the airways.* Am J Respir Cell Mol Biol, 2000. **22**(6): p. 649-56.

214. Davies, J.R., et al., *Mucins in airway secretions from healthy and chronic bronchitic subjects.* Biochem J, 1996. **313 (Pt 2)**: p. 431-9.

215. Sheehan, J.K., et al., *Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions.* Biochem J, 2000. **347 Pt 1**: p. 37-44.

216. Zuhdi Alimam, M., et al., *Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways.* Am J Respir Cell Mol Biol, 2000. **22**(3): p. 253-60.

217. Kaup, F.J., et al., *Ultrastructural findings in horses with chronic obstructive pulmonary disease (COPD). II: Pathomorphological changes of the terminal airways and the alveolar region.* Equine Vet J, 1990. **22**(5): p. 349-55.

218. Adler, K.B., et al., *Platelet activating factor stimulates secretion of mucin by explants of rodent airways in organ culture.* Experimental Lung Research, 1987. **13**(1): p. 25-43.

219. Dabbagh, K., et al., *IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo.* J Immunol, 1999. **162**(10): p. 6233-7.

220. Harkema, J.R. and J.A. Hotchkiss, *Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways.* Toxicol Lett, 1993. **68**(1-2): p. 251-63.

221. Hotchkiss, J.A., M.A. Stam, and J.R. Harkema, *Platelet activating factor stimulates rapid mucin secretion in rat nasal airways in vivo.* Experimental Lung Research, 1993. **19**(5): p. 545-57.

222. Rose, M.C., et al., *Model systems for investigating mucin gene expression in airway diseases.* J Aerosol Med, 2000. **13**(3): p. 245-61.

223. Vandenput, S., et al., *Airborne dust and aeroallergen concentrations in different sources of feed and bedding for horses.* Veterinary Quarterly, 1997. **19**(4): p. 154-158.

224. Gerber, V., et al., *Mucin genes in horse airways: MUC5AC, but not MUC2, may play a role in recurrent airway obstruction.* Equine Vet J, 2003. **35**(3): p. 252-7.

225. Harkema, J.R., et al., *Effects of an ambient level of ozone on primate nasal epithelial mucosubstances. Quantitative histochemistry.* American Journal of Pathology, 1987. **127**(1): p. 90-6.

226. Tatusova, T.A. and T.L. Madden, *BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences.* FEMS Microbiol Lett, 1999. **174**(2): p. 247-50.

227. Collins, D.W. and T.H. Jukes, *Rates of transition and transversion in coding sequences since the human-rodent divergence.* Genomics, 1994. **20**(3): p. 386-96.

228. Fitch, W.M., *The molecular evolution of cytochrome c in eukaryotes.* J Mol Evol, 1976. **8**(1): p. 13-40.

229. Stewart, F., et al., *Identification of the horse epidermal growth factor (EGF) coding sequence and its use in monitoring EGF gene expression in the endometrium of the pregnant mare.* J Mol Endocrinol, 1994. **12**(3): p. 341-50.

230. Janke, A., et al., *Phylogenetic analysis of 18S rRNA and the mitochondrial genomes of the wombat, Vombatus ursinus, and the spiny anteater, Tachyglossus aculeatus: increased support for the Marsupionta hypothesis.* J Mol Evol, 2002. **54**(1): p. 71-80.

231. Gerber, V., et al., *Airway inflammation and mucus in two age groups of asymptomatic well-performing sport horses.* Eq. Vet. J., 2003. **35**(5): p. 491-495.

232. Kirschvink, N., et al., *The use of cardboard bedding material as part of an environmental control regime for heaves-affected horses: in vitro assessment of airborne dust and aeroallergen concentration and in vivo effects on lung function.* Vet J, 2002. **163**(3): p. 319-25.