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Analysis of Asthmatic Lung Remodeling in Summer Pasture-Associated Recurrent Airway Obstruction

Claudenir Rodrigues Ferrari

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Analysis of asthmatic lung remodeling in summer pasture-associated recurrent airway
obstruction

By

Claudenir Rodrigues Ferrari

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

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Summer pasture-associated recurrent airway obstruction (SPARAO) is characterized by reversible airway obstruction resulting from airway hyper-reactivity to aeroallergens, mucus accumulation, and airway inflammation. These are key clinical features that are shared with human asthma, suggesting SPARAO's utility as an animal asthma model.

SPARAO affects horses maintained on pasture in conditions of high heat and humidity. Common in the southeastern United States, the cause of SPARAO is unknown, but is presumed to reflect reactivity to seasonally inhaled pasture-associated aeroallergens.

This investigation sought to identify well-characterized histopathological lesions of human asthma, collectively termed 'asthmatic remodeling', in lung tissue from horses with SPARAO. Two histological staining techniques were used: H&E and Movat's Pentachrome. Similar to chronic asthma, lung tissue from horses with SPARAO demonstrates statistically significant increases in airway smooth muscle, fibrosis, airway occlusion and inflammation, goblet cell hyperplasia, and remodeling of terminal bronchioles and elastin fibers.

Key words: SPARAO, summer pasture-associated recurrent airway obstruction, RAO, recurrent airway obstruction, histopathology of asthma, pentachrome stain

DEDICATION

I dedicate this dissertation to my wife, Flavia Ferrari, for being by my side during the great (and the not so great) days of pursuing a masters and a life abroad.

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CHAPTER I

INTRODUCTION

Recurrent airway obstruction (RAO), an asthma-like disease, is one of the most frequent respiratory diseases in horses,¹ and is generally recognized as a disease that occurs in mature horses worldwide that are housed in stalls.²⁻⁴ RAO is a naturally occurring allergic respiratory disorder characterized by exacerbations of acute airway obstruction followed by periods of remission.⁵ Airway obstruction results from bronchospasm, mucus accumulation, and airway inflammation.⁶ Risk factors associated with disease onset reflect increased aeroallergen/organic particle exposure and include inadequate air circulation, direct exposure to environmental aero-allergens (especially mold spores and pollen), exposure to stalls or barns with poor air quality, and the use of poor quality dusty or moldy hay.⁷ Conversely, disease remission is associated with allergen depletion in the environment of RAO affected horses.⁸

RAO is an economically significant disease of horses for which two syndromes have been described. Barn-associated RAO occurs more commonly in temperate climates,⁸ and summer pasture-associated recurrent airway obstruction (SPARAO) which affects horses maintained in pasture during summer in the southeastern United States and Great Britain.^{4,9} The incidence of RAO is rare in warm and dry climates such as in California and Australia.⁸ Barn-associated RAO is typically a disease of stabled horses during the winter.^{1,10} Though the stimuli eliciting barn-associated RAO are likely

complex and diverse, the disease is reliably triggered by challenge with organic molds that are found in hay.¹⁰ Clinical signs can be improved by reducing or eliminating dust and increasing ventilation in the stall.^{7, 10} Eliminating allergens that precipitate the clinical syndrome is essential to prevent and manage the disease.³

Unlike barn-associated RAO, SPARAO is reported in mature horses housed primarily on pasture during the spring and summer.¹¹ SPARAO affected horses experience a seasonal remission of clinical signs during the winter.¹¹ Inhalation of pollens and/or outdoor molds are thought to be the main cause of this summer condition.¹² However, clinical exacerbation of SPARAO is associated with increases in temperature, humidity, counts of fungal spores and counts of grass pollens.¹³

Pulmonary obstruction can involve central and/or terminal airways.¹⁴ While terminal airways provide little resistance to airflow in normal lung, investigations in human asthmatics suggest that small airways are responsible for the majority of gas trapping in the asthmatic lung.¹⁵ Though histopathologic lesions in large airways of human asthmatics are well characterized because they are easily accessible for endoscopic biopsy, histopathology of the terminal airways in human asthma is not well characterized.

Significantly, RAO possesses key clinical features of asthma¹⁶ including reversible airway obstruction, bronchial hyper-reactivity to aeroallergens, and chronic airway inflammation.^{2,6,7,13} The objective of this research is to identify histopathologic lesions that are common to the small airways of SPARAO affected horses and the small airways of human asthmatics.

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CHAPTER II
LITERATURE REVIEW

**Recurrent Airway Obstruction and Summer Pasture-Associated Recurrent
Airway Obstruction**

Recurrent Airway Obstruction (RAO, “heaves”) is a frequent equine pulmonary disease in the northern hemisphere.¹ It is an asthma-like condition of stabled horses characterized by reversible airway obstruction and airway inflammation that is initiated by inhalation of organic particles, especially from hay and bedding.² Horses with summer pasture-associated recurrent airway obstruction (SPARAO) also exhibit these asthma-like signs resulting in a syndrome that is clinically similar to barn-associated RAO. However, SPARAO occurs from spring until early fall in horses that are exposed to pasture in warm climates.³ Horses can, infrequently, be affected by both forms of the disease.⁴ Both recurrent airway obstruction and summer pasture-associated recurrent airway obstruction (RAO-SPARAO) are characterized by reversible airflow obstruction, airway neutrophilic inflammation, and mucus hypersecretion.^{3,5} Bronchial hyper-responsiveness is well documented in RAO⁴ and while presumed to occur in SPARAO, has not been experimentally documented.³ RAO and SPARAO occur as a clinical syndrome ranging from exercise intolerance and coughing to tachypnea, dyspnea, and wheezing. In both RAO and SPARAO, remission is observed when the allergen is removed from the horse environment.^{3,6} RAO and SPARAO have been previously referred to as chronic

obstructive pulmonary disease (COPD). However, in human COPD, airway obstruction is minimally reversible and airway hyper-reactivity is not a predominant facet of the disease. These and other differences make RAO and SPARAO more analogous to human asthma.²

Both RAO and SPARAO are considered to be caused by mixed hypersensitivity reactions to aeroallergens, especially molds and endotoxins. The precise immunopathological mechanisms and antigens responsible for causing the clinical syndrome in susceptible animals have not been fully elucidated.^{1, 7} Intradermal tests have confirmed hypersensitivity to numerous antigens, but the development of skin hypersensitivity does not assure a causative relationship for RAO.⁸ Allergens triggering SPARAO appear to be related to pasture, particularly pollen and outdoor molds^{3, 9} as evidenced by clinical worsening when exposed to pasture and improvement when horses with SPARAO are removed from pasture. It was reported that mature horses that are kept in pasture for more than 12 hours a day are more susceptible to develop SPARAO.^{10, 11} Both RAO and SPARAO are multifactorial diseases that result from genetic and environmental interactions. A poorly ventilated stall may trigger RAO, but a poorly ventilated stall in association with a genetically predisposed horse make the disease more severe.¹² Marti and collaborators (1991) demonstrated that offspring from horses with allergic pulmonary disease have an increased risk of developing chronic pulmonary hypersensitivity and this risk increases when both the sire and dam are affected by an allergic pulmonary disease.¹³

Although RAO is the most common chronic respiratory condition in stabled mature horses in the northern hemisphere,¹⁴ its occurrence is rare in warm and dry

climates such as California and Australia.⁶ Conversely, SPARAO has been reported in regions with warm and humid summers such as the southeastern United States and the United Kingdom.^{9,10,15} An epidemiologic study from 1998 that included 28 states from the United States demonstrated that 43% of winter stabled horses are RAO-affected.¹⁵ In addition, a study of risk factors for RAO in North American horses identified a considerably increased risk of RAO with increasing age. Horses greater than or equal to 7 years of age are 6 to 7 times more likely to develop RAO than younger horses.¹⁶ In 2007, a study of RAO prevalence in Great Britain demonstrated that 14% of the horses were RAO-affected. That same study concluded that increasing age and exposure to an urbanized environment are risk factors for RAO and suggested that air pollution was responsible for RAO in urban areas.¹⁷ A 2011 prevalence study also identified RAO as the most diagnosed veterinary pulmonary disease in Hungary.¹⁸ RAO is also more prevalent than SPARAO in Britain where maintaining horses on pastures as long as possible is recommended.¹⁹

Clinical signs of RAO result from airflow obstruction which occurs secondary to diffuse bronchoconstriction.¹⁴ The most common signs are flared nostrils, increased respiratory rate, anxiety, coughing, nasal discharge and reduced exercise tolerance. In the most severe cases a heave line, reflecting hypertrophy of the external abdominal oblique muscle, is evident.²⁰ In RAO, clinical signs are more expected during the winter while SPARAO clinical signs become more accentuated from July to September with a period of remission during the winter.¹¹

The pathogenesis of RAO and SPARAO have been gradually clarified by the continual application of new research tools. RAO has been investigated to a much greater

extent than SPARAO. Both innate and adaptive immunity contribute to the inflammatory process that is characteristic of RAO.²¹ Van der Haegen, et. al., (2005) demonstrated that IgE-positive cells (IgE+) are present in lung tissue of RAO-affected horses but the numbers of IgE positive cells did not differ between affected and control horses.²⁴ However, Dirscherl demonstrated that relative to basophils from control horses without RAO, basophils from horses with barn RAO were hypersensitized to a number of antigens, particularly molds of the genus *Mucor*. This work substantiates, in at least some cases of RAO, a role for IgE sensitized inflammatory cells as the gateway to antigen-specific recognition and initiation of inflammatory cascades.²²

Following allergen exposure, susceptible horses develop airway inflammation characterized by a massive neutrophil influx.²³ Transcription factor activation, particularly of NF- κ B, plays an important role in regulating cellular pathways responsible for the clinical syndrome.²⁴ Inflammatory cell activation by helper T-cells has been identified in RAO and the intensity of cytokine production has been correlated to disease chronicity.²³ Bronchoalveolar cells from RAO-affected horses have an increased expression of IL-4 and IL-5 mRNA, and decreased expression of INF- γ consistent with the Th-2 cytokine profile that has been often demonstrated in human asthma.²⁵ Tamarinde, et. al., (2006) further corroborated a Th2 polarization in RAO, demonstrating increased expression of TNF- α , IL-1 β and IL-8 in alveolar macrophages from RAO-susceptible horses relative to horses without RAO following sensitization. Expression of the anti-inflammatory cytokine, IL-6 was higher in the RAO susceptible groups after 6 hours of allergen challenge relative to horses that were not susceptible to RAO.²¹

Specific mediators of disease include, but are not limited to chymase, mucin and endothelin-1. Van der Haegen, et. al., (2005) demonstrated a relationship between high chymase+ mast cell (MCc) numbers in the bronchial wall and lung fibrosis, suggesting that MCc may be involved in the tissue remodeling associated with RAO.²⁶ Lugo, et. al., (2006) showed that the amount of stored mucin in goblet cells increases with airway inflammation in RAO.²⁷ Endothelin-1 (ET-1) is a powerful smooth muscle constriction and proinflammatory mediator that has been implicated in the pathogenesis of asthma. Benamou (1998) showed that the production and release of ET-1 is increased during a RAO exacerbation.²⁸ Costa corroborated this finding in SPARAO, demonstrating a significantly higher ET-1 concentration in plasma, BALF and pulmonary epithelial lining fluid from SPARAO affected horses during seasonal clinical exacerbation relative to their ET-1 concentrations in the season of disease remission (and relative to ET-1 concentrations of control horses during the period of clinical exacerbation). A 2008 study also revealed ET receptor expression to be higher in the peripheral lungs of SPARAO-affected horses relative to controls.²⁹ Cytological analysis of bronchoalveolar lavage fluid (BALF) is commonly employed to diagnose RAO and SPARAO. This method is simple, inexpensive and can be used for differentiating pulmonary infections and pulmonary inflammation.³⁰ Kutasi and collaborators (2001) concluded that historic analysis and clinical examination are not sufficient to diagnose RAO and SPARAO, yet veterinarians infrequently use auxiliary tests to confirm the disease. Also they demonstrated that the age of the horse, history, clinical examination, respiratory tract endoscopy and BALF are the most valuable test combination to diagnose pulmonary allergic diseases in horses.¹⁸ While measures of lung function including resistance and elastance are evaluated in

airway research involving RAO and SPARAO, the equipment necessary to evaluate lung function is not well suited to its use in field practice.¹⁴

Though RAO and SPARAO are chronic and progressive, medical therapy and environmental management can be effective in controlling the disease.³¹ However, because RAO and SPARAO are allergic diseases, eliminating the inciting antigens is crucial for effective response to therapy.¹¹ For both RAO and SPARAO, prognosis depends upon disease severity at the time of diagnosis and vigilant environmental management. Controlling airway inflammation and airway hyper-reactivity are important therapeutic goals.²⁰

Pulmonary Histopathologic Alterations in RAO-SPARAO

RAO and SPARAO share similar histologic lesions³². However, RAO has been studied with more frequency than SPARAO due its higher incidence and earlier discovery.

Mucus hypersecretion is an important feature of RAO and SPARAO which is present even during clinical remission of the disease.^{33, 34} Mucus accumulation in the airways contributes to exacerbation of clinical signs.³⁵ Costa, et. al., (2000), described mucus accumulation as the main change in small airways of SPARAO-affected horses³² and Polikepahad, et. al., (2008)³⁰ also suggested that the most prevalent histopathologic change in euthanized SPARAO-affected horses is mucus accumulation.

The inflammatory process in RAO and SPARAO is characterized by a predominance of neutrophilic infiltration which can vary in severity.^{10,26} A minor and occasional eosinophilic infiltration may also be present.³³ Lugo, et. al., (2006) demonstrated that in RAO-affected horses, this pulmonary inflammatory infiltrate is

located mainly in the peribronchiolar interstitial tissue, lamina propria and luminal surface of the airways.²⁷

Herszberg, et. al., (2006) used histomorphometry to demonstrate that RAO is characterized by an important increase of airway smooth muscle in small and large airways. Moreover, the same study demonstrated that this smooth muscle increase is associated with myocyte hyperplasia.³⁶ Mujahid, et. al., (2011) also used histomorphometry to show that increasing airway smooth muscle is a feature of SPARAO.³⁷ Additional research is warranted to clarify the degree to which airway smooth muscle proliferation contributes to airway remodeling and how this process occurs in RAO.

Airways of RAO affected horses exhibit goblet cell hyperplasia.³⁵ Furness, et. al., (2010) demonstrated the presence of goblet cells in noncartilaginous airways of RAO-affected horses, a histopathologic change correctly termed metaplasia and not hyperplasia, as they assumed.³⁸ Costa (2000) followed by Polikepahad (2008) also confirmed the presence of goblet cell metaplasia in noncartilaginous airways of SPARAO-affected horses.^{29,33}

Fibrosis was not considered an important histologic change in RAO-affected horses by Furness and collaborators (2010). They used an immunohistochemistry method to measure the distribution of collagen in noncartilaginous airways. As a result, they did not detect considerable collagen proliferation in RAO-affected horses.³⁸ Likewise, Costa, et. al., (2000) did not observe appreciable collagen or fibrosis in SPARAO lung.³²

Though the pulmonary histopathologic alterations in RAO and SPARAO are quite similar, equine allergic pulmonary disease is very complex. Therefore, more studies are

necessary to confirm the histopathologic findings in both conditions. Changes evident in asthma including elastic fiber proliferation and neovascularization have not been studied in RAO or SPARAO-affected horses, to date. In addition, some researchers have confused pathologic terminology, especially metaplasia, that has been frequently referred to as hyperplasia.

Histopathology of Asthmatic lungs

Macroscopic examination of a human asthmatic lung shows hemorrhagic changes, edema and hyperinflation of small airways.³⁹ Asthmatic lung presents various histopathologic alterations that are observed in cartilaginous (proximal) and noncartilaginous (distal) airways.³⁹ Tissue remodeling in the airways correlates to asthma severity.⁴⁰ In the human respiratory tract, air is distributed into more than 130,000 terminal airways (<2mm) and each terminal airway is subdivided into alveolar ducts and alveoli.⁴¹ Although noncartilaginous airways contribute to only a small proportion of airway resistance, pulmonary physiology is highly influenced by histologic alterations in noncartilaginous airways.⁴² Accordingly, in an asthmatic exacerbation, small airways may have an important influence on lung physiology.⁴³ Studies involving noncartilaginous airways have been limited because their small caliber makes these airways difficult to access. Nonetheless, transbronchial biopsy, morphometry, biochemical markers, flow-volume analysis and high-resolution imaging have been used to explore the peripheral lung and to improve the understanding of noncartilaginous airways in asthma.^{44, 45} Since residual volume of air caused by closure of terminal airways increases with age, Macklem⁴⁶ has suggested that terminal bronchioles are responsible for the majority of gas trapping in asthma.

Components of the chronic inflammatory process are suggested to be the main cause of architectural remodeling, irreversible airway obstruction and asthma severity⁴⁷.⁴⁸ Eosinophils are the main inflammatory cell in asthmatic lung and variation in the distribution of this inflammatory cell type has been noticed. Small airways show more eosinophilic accumulation within the airway wall whereas the density of eosinophils within the lumen is greater in the lumen of the large airways.⁴⁹ Consistent inflammation, especially eosinophilic, has an important role in determining asthma severity.^{50, 51} Lacoste, et. al., demonstrated an increased number of eosinophils in the blood, BALF and pulmonary biopsy of asthmatic patients. In addition, they also confirmed the presence of degranulated eosinophils only in asthmatic patients, when compared to COPD-affected and normal subjects.⁵² Carroll and collaborators (1996) demonstrated that the number of neutrophils increases and the number of eosinophils decreases in fatal asthma attacks of short duration, when compared to fatal asthma attacks of long duration. These findings have led to the proposal that asthma attacks are triggered by an inflammatory stimulus.⁵³ Ordonez and colleagues (2000) also concluded that neutrophils are the most common inflammatory cell in the airways in acute severe asthma.⁵⁴

Airway smooth muscle proliferation is one of the most important alterations in asthmatic lungs. It is associated with asthma severity^{48, 55} and it increases with age.⁵⁶ Prescott and collaborators (2004) demonstrated that airway smooth muscle proliferation is a feature of mild to moderate asthma. Further, they concluded that this proliferation is attributable to smooth muscle hyperplasia and that smooth muscle hypertrophy does not occur.⁵⁷

Goblet cell hyperplasia is an important characteristic of asthma. Ordonez, et. al., (2001) demonstrated that in mild asthma the hyperplastic goblet cells increase mucin storage while in moderate asthma the mucin is overstored and secreted. The same study suggested that goblet cell hypertrophy does not occur and secretion of mucin may be related to asthma severity.⁵⁸ Additionally, Aikawa and colleagues (1992) revealed that goblet cell hyperplasia is a prominent characteristic of patients who died from an asthma attack.⁵⁹

Histopathologic alteration in the airway epithelial tissue has been considered the major feature of airway remodeling in asthmatic lungs and the airway inflammatory process is also influenced by epithelial cells.⁶⁰ Loss of integrity, cellular detachment and squamous cell metaplasia are routinely observed, especially in the most severe cases.⁶¹

Subepithelial collagen deposition is a primary histopathologic alteration observed in asthmatic lungs.⁶² Chu, et. al., (1998) demonstrated that collagen deposition in large airways is a feature of asthma, but that it does not correlate to disease severity.⁴⁷ Opposing these results, Benayoun, et. al., (2003), demonstrated that collagen deposition and fibroblast accumulation in large airways are determinates of asthma severity.⁵⁵

Elastic fiber proliferation is a feature of asthmatic lungs that has not been correlated to disease severity. Carrol, et. al., (2000) performed a histopathologic study of small and large asthmatic airways and demonstrated that elastic fiber proliferation in fatal asthma is similar to nonfatal cases, whereas nonasthmatic control cases showed a decrease in the quantity of elastic fibers.⁶³

In summary, asthma is a heterogeneous condition. Some pulmonary histopathologic alterations correlate to asthma severity. Though inflammatory cell

composition has been extensively studied, characteristics including airway smooth muscle proliferation, collagen deposition and elastic fiber accumulation are in need of more extensive research.

Animal models of asthma

A variety of animal species have been used as asthma models. However, the histopathologic and immunologic complexities of asthma make⁶⁴ it challenging to identify an animal model capable of mimicking all the characteristics of human asthma. A perfect animal model for human asthma research should exhibit all the signs presented in the human disease, including cough, wheeze, bronchoconstriction, airway inflammation, airway hyperresponsiveness, airway remodeling and decreasing lung function. Animal models are very important to identify and test the efficacy and safety of new drugs.⁶⁵ Only horse and cat develop a spontaneous asthma-like syndrome that demonstrates a high level of similarity to the human condition.⁶⁶ Other animal asthma models have been induced in mouse, guinea pig, dog, sheep, and nonhuman primates. The characteristics of each animal model influence its suitability for investigating particular facets of asthma. Accordingly, the success of a given research hypothesis is largely influenced by the selection and suitability of the animal model that is investigated.⁶⁷

Murine model

The murine model is the most common animal asthma model.^{65, 68} Mice are inexpensive, breed rapidly, are available in genetically homogenous strains and are easily

handled. In addition, a great variety of transgenic strains are available for focused mechanistic research.

Transgenic mice are particularly useful to characterize key events and effector molecules that regulate airway inflammation.^{69, 70} However, the efficacy of mice models in studies of small-molecule therapeutics and their specific receptors is dubious, since the rodent receptors and signal-transduction pathways diverge significantly from those of humans.⁷¹ A vast number of different murine protocols are used for asthma studies but ovalbumin (OVA) is the most used antigen to induce asthma and intraperitoneal injection is the most frequent route of sensitization.⁷² This method is completely different from the naturally occurring human disease in which sensitization occurs via the respiratory tract. In addition, OVA does not trigger asthma in humans and allergens that trigger the human condition, such as pollen, dust, mites and molds, do not induce asthma in mice.^{68, 73}

Important differences between human asthma and the murine model have limited use of murine models in asthma research. Research mice are housed in an environment that is not similar to the human condition, the number of branching airways and submucosal glands are fewer in mouse lungs than human, and the airways are large relative to the size of the organism.⁷⁴ In addition, airway hyperresponsiveness in humans is immensely greater than in the mouse. Human asthma is also more restricted to the airways while the murine condition extends to the lung parenchyma and pleura.⁷⁵ In contrast to human asthma, the murine model also does not show airway smooth muscle increase or a late asthmatic response.⁷⁶⁻⁷⁸ Another disadvantage is the fact that prolonged antigen exposure inhibits eosinophilic inflammation and prevents further exacerbations triggered by the same antigen. Moreover, airway hyperresponsiveness is also inhibited by

prolonged antigen exposure.^{79, 80} The murine model also fails to simulate chronic inflammation and epithelial alterations, both important characteristics of human asthma that are not present in rodents. Also, the inflammatory process and the detected airway hyperreactivity cease after removing the allergen exposure. These collective limitations have led to questions regarding the applicability of the mouse model.^{70, 81}

Recently, several alternative murine models have been tested with the goal to better simulate the histopathological alterations and immunological processes that occur in human asthma in order to make the mouse more useful in asthma research.^{70, 82-86}

Guinea Pig model

The guinea pig has been recognized as an important model for human asthma studies. They are small, docile, inexpensive and easily handled. Moreover, this model presents a histological and clinical asthmatic condition similar to human asthma.⁸⁷ Guinea pig asthma exhibits airway remodeling, inflammation and hyperresponsiveness.⁸⁸⁻⁹¹ Accordingly, the guinea pig is well suited for airway hyperresponsiveness research and has been extensively employed in investigations of airway hyperresponsiveness.⁹²

In contrast to mice and rats, the guinea pig is capable of developing early and late asthmatic responses and during the late response, eosinophilic and neutrophilic inflammation are observed.⁹¹ A recent asthma study with guinea pig models also revealed that airway eosinophilia and airway hyperresponsiveness may occur in association with each other, but a causal association between these factors was not demonstrated.⁹³

The use of the guinea pig model in asthma research has important limitations. This model has a scarceness of genetically modified animals, lack of appropriate species-specific reagents such as antibodies and cytokines, and it overreacts to histamine

challenge when compared to the human reaction.^{64, 94} Additionally, in contrast to the spontaneous asthmatic development in humans, the asthmatic condition in guinea pigs requires induction by antigen sensitization.^{88, 89, 93-96}

Feline Model

Due to the spontaneous development of asthma in cats, the feline model is considered an interesting and valuable animal model for asthma research.⁹⁷ A variety of immunologic, physiologic and pathologic alterations that resemble human asthma are present in feline asthma.⁹⁸ Prevalence of feline asthma syndrome is unknown because a large number of affected cats are not reported by their owners.⁹⁹ The correct diagnosis of asthma is made by analysis of clinical signs and radiographic findings, including chronic cough, examination of bronchial wall thickening by radiography, high eosinophil counts in cytology of tracheobronchial secretions and history of rapid recovery from tachypnea after using bronchodilators. These are also characteristics found in eosinophilic human asthma.¹⁰⁰

Especially under chronic exposure to allergen, the feline model is capable of representing important characteristics of human asthma such as eosinophilic airway inflammation, airway hyperresponsiveness, airway remodeling, airway smooth muscle thickening, goblet cell hyperplasia, and allergen-specific IgE.^{98, 104} In addition, acute clinical signs of the human condition, such as cough, wheezing and tachypnea, are also reported.^{64, 98, 99, 101-103}

Disadvantages of using cats as an animal model for human asthma include limited numbers of interventions that can be tolerated due to their small body size.¹⁰³ Molecular

tools are also poorly developed for feline models,¹⁰⁴ and ethical issues have decreased asthma research in the feline model.

Canine Model

The dog has been used for asthma research involving long-term exposure to inhaled irritants and for pharmacologic testing.¹⁰⁵ As an induced asthma model, ragweed pollen and *Ascaris suum* antigen are the most common allergens used to sensitize dogs and to trigger the asthma phenotype. Following sensitization, dogs develop important characteristics of human asthma such as mucus hypersecretion, cough, airway hyperresponsiveness and airway inflammation.¹⁰⁶⁻¹⁰⁸ As in human asthma, both large and small airways constrict in the dog asthma model.¹⁰⁵

Unlike other animal models, the dog has been employed as a spontaneous model of exercise-induced asthma. Dogs are active animals and they exercise naturally. Davis, et. al., described a significant prevalence of airway inflammation after exercise in Alaskan sled dogs raced at temperatures between 0°C and -40°C.¹⁰⁹ In another study involving exercise-induced asthma, Freed and collaborators used dogs to demonstrate that exercise in a dry air environment can cause an airway constrictor response.¹¹⁰

Though useful as an animal asthma model, particularly for exercise-induced asthma, the canine model also has some disadvantages. Unlike human asthma, the late-phase response is not common in the canine model.¹⁰⁸ Dermatitis is a much more common manifestation of allergy in dogs.¹¹¹

Sheep Model

Sheep are employed as induced animal asthma models. This model provides the ability to repeatedly measure pulmonary function with precision.^{112, 113} Significantly, the methodologies and equipment used for detailed respiratory research in humans can also be used for sheep model research.¹¹⁴

The respiratory systems of sheep and humans share certain commonalities. Lung function similarities include similar physiological airflow, resistance, breathing rates, tidal volumes, and compliance. The airway similarities include similar alveoli, airway size, cartilage distribution, sensory nerves, capillaries, collagen and effect of histamine. In addition, cough reflex, wheeze, bronchial glands and goblet cells are also present.⁶⁷ Snibson and collaborators demonstrated that sheep repeatedly challenged with house dust mites can reproduce a remodeling-like alteration that is very similar to the remodeling in human lung.¹¹⁵

The use of the sheep model for asthma research has some limitations, including lack of inbred and genetically manipulated sheep, lower availability of appropriate immunologic reagents and high cost associated with purchase and husbandry.⁶⁷ However, the recent introduction of improved methods for bioengineering of transgenic animals is sure to improve the availability and application of transgenic sheep in asthma research.

Equine Model

The horse possesses certain advantages as an animal model of asthma. Horses spontaneously develop recurrent airway obstruction, an asthma-like disease that entails an exacerbation remission cycle and is triggered specifically by aeroallergens including hay and barn dust.^{6, 14, 66} Similarly to the human disease, horses also present clinical signs

such as dyspnea, coughing and wheezing.³ The horse RAO model exhibits the essential characteristics of human asthma, including airway remodeling, airway obstruction, bronchial hyperresponsiveness, smooth muscle proliferation, airway fibrosis, Goblet cell hyperplasia, airway inflammation and mucus hypersecretion.^{27, 34-37, 66, 116, 117} Airway inflammation in RAO-affected horses is predominantly neutrophilic; however, Polikepahad, et. al., (2008) demonstrated that, as in human asthma, some RAO-affected horses can present a moderate eosinophilic airway infiltration.²⁹ It is also known that severe cases of human asthma commonly have neutrophilic inflammation as is characteristic of horses with RAO.^{53, 54}

Despite being considered one of the most complete animal models for asthma research, the equine model also has some limitations. Immunological reagents are limited for use in the horse and the horse is best suited to model asthma phenotypes that are dominated by neutrophils.^{102, 119} Quite significantly, the expense of keeping a horse for research is very high. As a large animal, horses require ample space, a large amount of food, and regular veterinary care.

Nonhuman Primate Models

Monkey models are the most suitable asthma model for investigating immune regulation and to test new immunomodulatory therapies in asthma. The “toolbox” used for analysis of immune response in primates is amply greater than in other models and the genetic similarity between human and nonhuman primates may provide a very similar course of the disease.¹¹⁸ Moreover, the innervation of airways in monkeys is similar to that of human.¹⁰⁴ Three species of nonhuman primates have been used in research: *Saimiri sciureus* (squirrel monkey), *Macaca mulatta* (rhesus monkey), and *Macaca*

fascicularis (cynomolgus monkey).¹¹⁹ Using house dust mites, a classic human allergen, Schelegle, et. al., demonstrated that sensitized rhesus monkeys exhibit the critical characteristics of human asthma. Namely, the presence of IgE-positive cells in the tracheobronchial airway walls, airway obstruction, airway eosinophilic inflammation, airway hyperresponsiveness, goblet cell hyperplasia, epithelial hypertrophy, and thickening of the basement membrane zone were confirmed. Additionally, coughing, dyspnea and decreased arterial oxygen saturation were demonstrated.¹²⁰ In another study involving infant rhesus monkeys, the incidence of smooth muscle hypertrophy and hyperplasia were substantial in monkeys chronically sensitized to house dust mites.¹²¹

There are several limitations of using nonhuman primates as an animal model. Most significantly, the expense of purchasing the animals, food, medical care and housing make use of large numbers of subjects very difficult. Further, studies involving primates are chronologically extensive⁶⁸ and many ethical issues arise with the use of captive primates.

Summary

Asthma is an economically significant human disease with high morbidity. The spontaneous asthma-like disease of horses, termed RAO presents several improvements over mouse models. To date these similarities have been best characterized in the stall associated form of RAO that affects horses housed indoors in temperate regions.^{2,6,7} Most significantly, RAO models the clinical hallmarks of asthma: chronic airway inflammation, bronchial hyper-responsiveness, and reversible airway obstruction.^{2,5-8} The ability of RAO to model chronic neutrophilic inflammation is noteworthy because only the horse asthma model possesses airway neutrophilic inflammation and this

inflammatory infiltrate correlates to the most severe asthma phenotypes.¹²²⁻¹²⁴ Horses with RAO also develop increased airway smooth muscle³⁷ which is a histologic change of chronic airway remodeling in asthma that is not well modeled in any induced asthma model.¹²⁵⁻¹²⁸ The purpose of this work is to investigate the histologic features of SPARAO in order to determine if SPARAO possesses key features of remodeling that are identified in human asthma.

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CHAPTER III

MODIFICATION OF MOVAT'S PENTACHROME I STAIN

Abstract

Movat's pentachrome I is a stain technique with the ability to allow clear visualization of elastic fibers, muscle, collagen, mucin, ground substance, cytoplasm and nucleus in a single histologic section. As originally described, the stain requires process times of 8-22 hours for consistent staining outcomes. The objective of this investigation was to develop a modification of Movat's pentachrome I stain that reduces the process time and increases staining quality and consistency. The Movat's pentachrome I stain protocol was modified primarily by employing oven fixation of alcian blue and replacing overnight fuchsin staining with an 8 minute crocein scarlet and fuchsin stain in acidic conditions. Case sections from equine lung demonstrate the stain's efficacy. This protocol reduces prior stain times exceeding 20 hours to less than 2 hours, and provides clear color contrast of collagen in yellow, elastic fibers in black, ground substance in indigo, muscle in red, and mucin and cartilage in bright blue. Nuclei and cytoplasm stain dark blue and pink, respectively. This protocol's ability to clearly differentiate multiple histological features of a tissue in one stain technique, combined with the substantial decrease in the time to complete the staining procedure, allow for increased efficiency and accuracy during histologic evaluation of tissues.

Introduction

Movat's pentachrome I is a stain technique published by Movat in 1955.¹ This method provides color contrast of the tissue structures, exhibiting cytoplasm in pink, collagen in yellow, elastic fibers in black, ground substance in purple, mucin and cartilage in blue, nuclei in dark blue and muscle in red. In spite of its potential, this stain is infrequently used due to lack of familiarity among pathologists, researchers, and histology technicians, and absence of detailed and well explained protocols. In addition, the stain is intricate, sensitive to protocol errors, time consuming (in excess of 20 hours), can be inconsistent. Russell², Clifford³, Garvey⁴, and Schmidt⁵ modified Movat's technique but these protocols remained time consuming or have provided inconsistent staining characteristics. Elbadawi developed a hexachrome stain technique that is very similar to Movat pentachrome I stain.⁶ This technique had reduced time, but visualization of histologic structures was still unsatisfactory.

The purpose of this work was to refine a modification of Movat's pentachrome I stain that is easily prepared and can be executed in under 2 hours, even by inexperienced technicians. Lung sections are especially well suited to staining by Movat's pentachrome I because the lung tissue will display all the color contrasts provided by this technique.

Materials and Methods

To demonstrate this modification's staining utility, equine lung tissue sections collected at necropsy were used for the procedure. Tissues were fixed in 10% neutral formalin, embedded in paraffin and sectioned at 5 μ m. Sections were placed in xylene for 2 minutes followed by absolute ethanol for 2 minutes and then distilled water for 1 minute. The stock solutions utilized in this protocol include stock solutions A, B, C, and

D, working elastic solution, and mucin stain, ammoniated ethanol, plasma stain, polyacid, and fiber stain. The formulations of these solutions are detailed in appendix A. Sections were placed in mucin stain for 20 minutes, washed in tap water for 3 minutes, placed in ammoniated ethanol for 10 min at 58°C, and rinsed in running tap water for 5 minutes. Next, sections were stained in working elastic solution for 20 minutes and rinsed in running tap water until collagen was visualized as clear and elastin fibers were prominent. Slides were placed in plasma stain for 12 minutes followed by 0.5% aqueous acetic acid for 30 seconds. The slides were differentiated in polyacid until collagen appeared clear and ground substance appeared indigo as visualized using light microscopy. In our experience, this is a time sensitive step which may require 5-20 minutes of incubation. Slides should be examined at 5 minutes and observations repeated at 1-3 minute intervals depending upon the intensity of blue staining remaining in the ground substance. Slides were then rinsed in 0.5% aqueous acetic acid for 30 seconds, placed in absolute ethanol for 2 minutes, incubated in fiber stain for 15 minutes and rinsed using two changes of fresh absolute ethanol (1 min/rinse). Slides were placed in xylene for 3 minutes and coverslip mounted using standard resinous mounting medium.

Results

The stain protocol was routinely completed in one hour and forty five minutes. This modification of the Movat's pentachrome I stain clearly differentiated cytoplasm in pink, collagen in yellow, elastic fibers in black, ground substance in purple, mucin and cartilage in blue, nuclei in dark blue and muscle in red (Figures 1, 2 and 3)

Discussion

Certain features of the staining protocol were found to be particularly important to a successful outcome. The success of the mucin stain depends directly on ammoniated ethanol incubation. The temperature of the ammoniated ethanol must be exactly 58°C. The working elastic solution that is used must be made fresh for the stain. If not fresh, incomplete elastic fiber staining will occur and the elastic fibers will stain purple rather than black. Perhaps the most significant portion of the procedure follows the staining of elastic fibers. It is critically important to assure, with the aid of a microscope, that the collagen has cleared of staining before proceeding.

This modification of the Movat's pentachrome I stain improves the prior stain protocols¹⁻⁵ which require 20-22 hours to complete. This protocol consistently provides clear differentiation of elastic fibers, muscle, collagen, mucin, ground substance, cytoplasm and the nucleus, whereas consistency of staining is problematic in our hands with these prior protocols. This modification of the Movat's pentachrome I stain technique is very useful for showing the most important tissue structures in lung, enabling a clear comparison between normal and affected lung. This improved protocol for Movat's pentachrome I stain could be easily prepared and executed, even by inexperienced histology technicians.

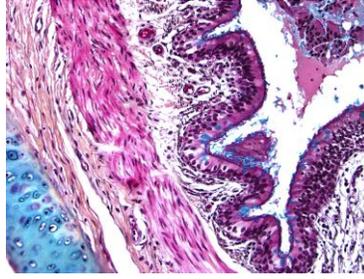


Figure 3.1 Lung, equine. Stained by Modified Movat's pentachrome I stain, 200X.

Cytoplasm of bronchial respiratory epithelium is pink to red, nuclei are dark blue, mucus in goblet cells is bright blue, hyaline cartilage is bright blue, smooth muscle is red, collagen is yellow and elastin fibers are black.

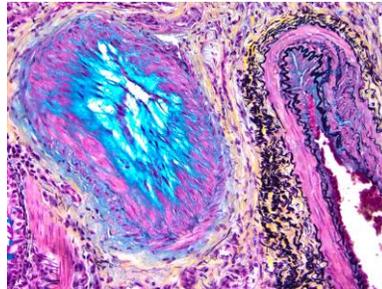


Figure 3.2 Lung, equine. Stained by Modified Movat's pentachrome I stain, 200X.

In muscular and elastic arteries, smooth muscle is red, elastic fibers in the arterial wall and adventitia are black, collagen is yellow, and mucinous matrix in the intima and tunica muscularis of arteries is bright blue.

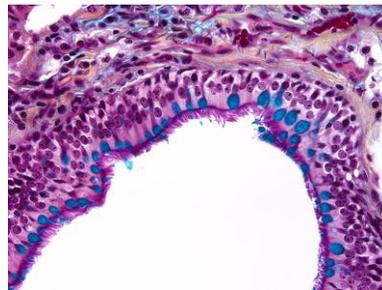


Figure 3.3 Lung, equine. Bronchus in equine lung. Stained by Modified Movat's pentachrome I stain, 400X.

Cytoplasm of bronchial respiratory epithelium is pink to red, nuclei are dark blue, mucus in goblet cells is bright blue, extracellular mucinous matrix is light blue, smooth muscle fibers are red and collagen is yellow.

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CHAPTER IV
FREQUENCY OF HISTOLOGIC LESIONS OF ASTHMATIC AIRWAY
REMODELING IN HORSES WITH SUMMER PASTURE ASSOCIATED
RECURRENT AIRWAY OBSTRUCTION

Introduction

The pathogenesis of asthma has been studied in a variety of animal models including rodents, sheep, cats, dogs, and primates¹. None of these models simultaneously demonstrate key clinical correlates to human asthma severity – namely, increased airway smooth muscle² and airway neutrophilia.^{3,4} The induced asthma phenotype of rodents is also a transient phenotype where, even in ‘chronic’ models, antigen sensitization cannot be maintained, suggesting that rodent models are progressing toward a state of antigen tolerance which is not characteristic of asthma.⁵⁻⁸ Larger animal models including dog, cat, sheep, swine, horse and monkey have distinct advantages.^{1,9} Facets of asthma that are not effectively modeled in rodents, including reversible airway hyper-responsiveness to aeroallergen challenge that is chronic and progressive, increased airway smooth muscle, and airway neutrophilic inflammation have been demonstrated to be facets of an equine model of asthma.^{10, 11,12} Large animals are well suited to repeated sampling and their physiological variables, including evaluation of pulmonary function, are more accessible for examination and more relevant to the physiology of the human lung than rodents.¹ In addition, tissue and body fluids are easily collected and the higher quantities

of material facilitate multiple analyses. Of the two spontaneous animal asthma models, the horse and cat, only the horse has non-eosinophilic airway inflammation; feline asthma is characterized by eosinophilic airway inflammation.¹

Recurrent airway obstruction (RAO), also termed equine ‘heaves’, is the horse counterpart of human asthma.¹² RAO is one of the most frequent lung diseases in horses¹³ and is a naturally occurring allergic respiratory disorder characterized by onsets of acute airway obstruction followed by periods of remission.¹⁴ RAO is described in two disease variants. The variant designated as RAO or ‘heaves’ is commonly described affecting horses in temperate areas of the world, particularly in the northern hemisphere, and is associated with indoor housing and exposure to dust and moldy hay.¹² The second form is summer pasture-associated recurrent airway obstruction (SPARAO), which occurs in the warm and humid summer in the southeastern United States, and United Kingdom.¹⁵ Mississippi and Louisiana are two of the 22 US states in which the prevalence of human asthma has increased since 2009,¹⁶ a distribution that matches the region of the United States in which horses with SPARAO manifest asthma-like signs on pasture in the summer. Reversible airflow obstruction, bronchial hyperresponsiveness, airway neutrophilic inflammation and mucus hypersecretion are facets of asthma that have been well described in the stall associated form of RAO.^{12, 17} These facets of asthma have also been documented in SPARAO.^{10, 11, 18} Similar to asthma, during RAO episodes, horses will present with coughing and exercise intolerance, and progress to tachypnea, dyspnea and wheezing.¹⁵ In both the indoor and pasture associated forms of RAO, clinical remission is observed when the aeroallergens associated with the triggering environment are removed.¹⁹

In normal lung, terminal airways have a small contribution to airway resistance.²⁰ In an asthmatic, lung terminal airways have a major impact on respiratory function because their absence of cilia and lower gas velocity decrease the ability to remove secretions that contribute to asthmatic airway obstruction.²¹ Histopathologic studies have established that the asthmatic process involves both central and terminal airways²² and measurement of the residual volume of air that is trapped during closure of terminal airways suggests that they are responsible for the greatest gas trapping in asthma.²³ Due to ease of access, much of pulmonary research has focused on large airways while terminal airways are less frequently investigated.²⁴ The histologic lesions of asthma, termed airway remodeling, correlate to advancing asthma and degrees of airflow limitation that are not reversible.²⁶ These lesions include airway obstruction, goblet cell hyperplasia and metaplasia, mucus hypersecretion, thickening of airway epithelium, smooth muscle proliferation and airway fibrosis.²⁵⁻⁴⁴ These lesions have also been described in horses with RAO.³⁸⁻⁴⁴ Airway neutrophilic inflammation is common in adult asthma⁴⁵ and the horse presents the only spontaneous asthma model with neutrophilic airway inflammation.¹ To document similarities between this spontaneous equine model and human asthma, the goal of this study was to objectively validate the presence and severity of characteristic histopathologic lesions of asthma in the terminal airways of SPARAO-affected horses.

Materials and Methods

Animals and Sample Collection

Archived lung tissue collected between 1996 and 2004 were used in this study. Animal protocols from which these tissues were derived were approved by the

Animal Care and Use Committee of Louisiana State University. Twenty-three horses were included in this study. Fifteen SPARAO-affected horses included 9 American Quarter Horses, 1 American Paint Horse, 2 Thoroughbreds, 1 Appaloosa, and 2 mixed breed horses. The SPARAO horses, 9 females and 6 geldings, ranged from 10 to 27 years of age (mean (x) \pm standard deviation (SD), 15.4 ± 5.3 years). Eight clinically normal horses included 3 American Quarter Horses, 1 American Saddlebred, 3 Thoroughbreds, and 1 Appaloosa/Thoroughbred cross. Control horses, 3 females and 5 geldings, ranged from 7 to 22 years of age ($x=15.6 \pm 5.3$ years). The diagnosis of SPARAO was based on the history of recurrent obstructive respiratory disease following exposure to summer pasture in the southeastern United States. This was substantiated by the following findings during summer that abated during cooler seasons: pronounced expiratory wheezes throughout the lung fields; tracheal rattle, cough, nostril flaring and prominent abdominal lift; increased clinical scores of respiratory effort (CSRE); and the presence of airway inflammation as documented by cytologic analysis of bronchoalveolar lavage fluid (BALF).¹⁰ The clinical score of respiratory effort, is based upon subjective scoring of nostril flare and abdominal lift on a scale of 1-4 and scores ≥ 4.5 substantiate dyspnea in horses with SPARAO.¹⁰

Twelve affected horses were euthanized during clinical exacerbation, eleven had wheezes upon auscultation of the lungs during tidal breathing. Their assigned CRSE ranged from 4.5 to 7.5 ($x = 5.4 \pm 0.8$), and cytologic evaluation of BALF confirmed a predominance of non-degenerate neutrophils, ranging from 10 to 90%, ($x = 60 \pm 26 \%$), and ΔPpl_{max} (maximal change in pleural pressure during respiration) ranging from 12 to 58 mm H₂O ($x = 29 \pm 13$). Three affected horses were euthanized during clinical

remission. None of these three horses had abnormal lung sounds upon auscultation of the lungs with a re-breathing bag. During remission, SPARAO horses had assigned CRSE of 3 ($x = 3 \pm 0$), and cytologic evaluation of BALF revealed neutrophils ranging from 1 to 3% ($x = 1.7 \pm 1.2 \%$), and ΔPpl_{max} ranging from 1 to 7 mm H₂O ($x = 4.7 \pm 3.2$). All non-affected horses had normal bronchovesicular sounds upon auscultation of the lungs with a re-breathing bag, assigned CSRE ranging from 2.0 to 3.0 ($x = 2.3 \pm 0.5$), and between 1% to 10% neutrophils ($x = 6 \pm 4 \%$) evident via cytologic evaluation of BALF.

All horses were euthanized by intravenous administration of sodium pentobarbital (100 mg/kg, IV) at least 24 hours after BALF sample collection. Gross postmortem evaluation of the lungs was performed and the lungs were removed from the thoracic cavity. Of the twelve affected horses euthanized during clinical exacerbation, eleven had overinflation of the lungs evidenced by failure to collapse when the thoracic cavity was opened. The lungs of all of the SPARAO-affected horses euthanized during remission and all non-affected horses collapsed when the thoracic cavity was opened and none appeared overinflated. Two specimens measuring 3x3x3 cm were collected from the right apical, left apical, right diaphragmatic, left diaphragmatic and accessory lung lobes, respectively. Each specimen was selected to contain at least 1 bronchus with diameter between 4 to 8 mm. Immediately after collection, specimens were immersed in aqueous buffered zinc formalin for 24 hours prior to routine processing, embedding in paraffin, and sectioning.

Tissue preparation and histological Staining Techniques

Histologic sections were cut at 5 μ m and stained with hematoxylin-eosin stain or a modification of Movat's Pentachrome I Stain as described in Chapter 3. Movat's

pentachrome I stain provides color contrast that enables visualization of elastic fibers, muscle, collagen, mucin, ground substance, cytoplasm and nucleus in a single histologic section. Cytoplasm is readily evident by its conventional pink staining, collagen stains yellow, elastic fibers black, ground substance indigo, the nucleus dark blue, muscle red, and mucin and cartilage bright blue.

All non-cartilaginous airways were identified on each slide and labeled. Fifteen non-cartilaginous airways were randomized and evaluated for histologic evidence of asthmatic airway remodeling including increases in airway smooth muscle, peribronchiolar fibrosis with collagen, peribronchiolar elastin fibers, airway occlusion by mucus or inflammatory cells, airway adventitial inflammation, and goblet cell hyperplasia and metaplasia. Initial review of lung sections from horses with SPARAO revealed a complex cellular disorganization at the level of the terminal bronchus. This lesion, which we have named terminal bronchiolar remodeling, is characterized by varying degrees of extension of smooth muscle, collagen and elastic fibers into surrounding alveolar ducts, alveoli and alveolar septal walls together with varying degrees of terminal bronchiolar epithelial hyperplasia. These seven histologic parameters were evaluated and scored in each airway as 0 (normal), 1 (mild), 2 (moderate) and 3 (severe) according to criteria listed in Table 1

Statistics

A mixed effects logistic regression model was used to describe the effect of independent variables on the probability of reporting the histologic outcome using PROC GLIMMIX (SAS 9.1 for Windows, SAS Institute, Inc., Cary, NC). Histological parameter scores were dichotomized with scores of 0 considered negative (0), and scores

of 1, 2 or 3 considered positive. The independent variables in the model were disease status (SPARAO vs control), lung lobe, and age where horses were grouped as ≤ 15 years and >15 years. The hierarchical structure of the data was accounted for by including lung lobe (horse ID) and horse ID as random effects. Pairwise comparisons of lobe effects were determined using least squares means. The threshold for statistical significance was $p < 0.05$.

Results

Table 2 summarizes the comparisons of the 7 histologic parameters by disease status and age. The frequency of all seven histologic parameters was significantly increased in horses with SPARAO relative to control horses. Increases in airway smooth muscle (OR=2.5, $p < 0.001$), terminal bronchiolar remodeling (OR=3.7, $p < 0.0001$), goblet cell hyperplasia/metaplasia (OR=37.6, $p < 0.0001$), and peribronchiolar elastin fibers (OR=4.2, $p < 0.001$) were highly significant in SPARAO horses. Increases in peribronchial fibrosis (OR=3.8, $p = 0.01$), airway occlusion by mucus and inflammation (OR=4.2, $p = 0.04$), and airway adventitial inflammation (OR=3.0, $p = 0.01$) also met the criteria established for statistical significance.

The frequency of histologic lesions was not significantly different between age groups for increased airway smooth muscle (OR=1.5, 0.9-2.6; $p = 0.11$), airway occlusion by mucus and inflammation (OR=2.5, 0.7-9.3; $p = 0.17$), airway adventitial inflammation (OR=1.3, 0.5-3.0; $p = 0.57$), and goblet cell hyperplasia/metaplasia (OR=0.6, 0.4-1.0; $p = 0.6$). Relative to younger horses, older horses had significantly greater peribronchial fibrosis (OR=2.7; $p = 0.04$), terminal bronchial remodeling (OR=2.4; $p = 0.0004$), and peribronchiolar elastin fibers (OR=2.9; $p = 0.004$).

Significant differences in the frequency of increased airway smooth muscle ($p=0.001$), terminal bronchiolar remodeling ($p=0.0001$), and peribronchiolar elastin fibers ($p<0.0001$) were evident between lung lobes. In all pairwise comparisons between the accessory, left apical and right apical lung lobes, the frequencies of increased airway smooth muscle ($p>0.70$), terminal bronchiolar remodeling ($p>0.6$), and peribronchiolar elastin fibers ($p>0.08$) did not differ significantly between lung lobes. However, all pairwise comparisons of the left or right diaphragmatic lobe to either the accessory, left apical or right apical lung lobes identified statistically significant increases in airway smooth muscle ($p\leq 0.01$) and terminal bronchiolar remodeling ($p<0.05$). The frequency of increased airway smooth muscle was slightly higher in the right diaphragmatic lobe than the left diaphragmatic lobe, but was not significant ($p=0.44$). Frequency of terminal bronchiolar remodeling was significantly higher in the right diaphragmatic lobe than the left diaphragmatic lobe ($p=0.02$). In the case of peribronchiolar elastin fibers, pairwise comparisons of the left or right diaphragmatic lobe to either the left apical or right apical lung lobes identified a significant increase in peribronchiolar elastin fibers ($p\leq 0.017$) in the left and right diaphragmatic lung lobes. The frequency of increased peribronchiolar elastin fibers was slightly higher in the left diaphragmatic lobe than the right diaphragmatic lobe but this difference was not statistically significant ($p=0.09$). The frequency of peribronchiolar elastin fibers in the accessory lobe was not significantly different from the right diaphragmatic lung lobe ($p=0.10$). The accessory lobe had fewer peribronchiolar elastin fibers than the left diaphragmatic lobe and this difference was highly significant ($p<0.0001$).

Discussion

Asthma is a clinical syndrome characterized by variable bronchial hyper-responsiveness leading to respiratory distress and airway obstruction. Small airways are now recognized to have a major impact on respiratory function in asthma. Measurements of the residual volume of air that is trapped during closure of terminal airways suggests that they are responsible for the greatest gas trapping in asthma.²³ This has been attributed to the absence of cilia and have lower gas velocities in small airways that impair removal of respiratory secretions.²² Impaired removal of respiratory secretions from small airways and the resulting obstruction of these airways is a major contributor to impaired gas exchange in asthma because small airways link regions of pulmonary gas exchange to the larger conducting airways.

Despite physiologic evidence for the importance of small airways in asthma, and histopathologic evidence that the asthmatic process involves both central and terminal airways,²² small airway pathology in asthma has not been as extensively investigated as the pathology of larger airways. This reflects the relative ease of accessing the larger airways for bronchoscopic biopsy. In this investigation the characteristic histologic components of asthma pathology, collectively termed ‘remodeling’ were evaluated in smaller cartilaginous and non-noncartilagenous airways from horses with SPARAO, a proposed equine model of non-eosinophilic asthma. These lesions include smooth muscle hypertrophy or hyperplasia, peribronchiolar fibrosis with collagen deposition, airway occlusion by mucus or inflammatory cells, airway adventitial inflammation, and goblet cell hyperplasia or metaplasia. It is generally accepted that airway remodeling is closely related to the progression and severity of asthma.²⁹ This investigation also identified

complex cellular disorganization at the level of the terminal bronchus characterized by varying degrees of extension of smooth muscle, collagen and elastic fibers into surrounding alveolar ducts, alveoli and alveolar septal walls and accompanied by varying degrees of terminal bronchiolar epithelial hyperplasia. To our knowledge, this is first description of this lesion which has been termed terminal bronchiolar remodeling. Finally, the presence of increased elastic fibers in the airway, a histologic finding that is increased in severe fatal asthma, was also characterized.⁴⁵ The use of a modified pentachrome stain in our research was extremely important for providing a detailed view of the most important microscopic changes in lungs. The stain modification developed in the course of this research significantly shortened the duration of the staining protocol and enabled the evaluation of multiple tissue components within a single stained section, making it of great value for histopathologic lung research.

Increases in airway smooth muscle have been previously identified in SPARAO.⁴² In our study, an increase in smooth muscle in the small airways constituted a highly significant difference between SPARAO horses and controls. Horses with SPARAO were 2.5 times more likely to have increased airway smooth muscle than control horses. Our statistical model included disease, age and lung lobe as independent variables, such that this odds ratio is not biased by age. Further, the frequency of increased airway smooth muscle was not statistically significantly different between age groups. Similar to our results, Herszberg and colleagues⁴¹ demonstrated that smooth muscle proliferation is much more accentuated in small airways than in large airways in horses with barn associated RAO. In association with staining that documented proliferation of smooth

muscle cells, these authors concluded that airway smooth muscle hyperplasia is an important factor in airway obstruction and hyper-responsiveness in barn associated RAO.

In human asthmatics, investigations of airway smooth muscle are primarily derived from large airways which are accessible using endoscopic biopsy techniques. Proliferation of airway smooth muscle in large airways is greater in subjects with severe asthma, as compared with the less severe forms of the disease,^{46, 47} indicating that airway smooth muscle hyperplasia is the key structural change in the progression from less severe to more severe forms of human asthma. Mauad and colleagues recently reported increased airway smooth muscle in small airways of fatal asthma in young patients (mean age 32 years), further supporting a role for increased airway smooth muscle as a marker of advancing asthma severity.⁴⁸ In this investigation of small airways from a spontaneous equine model of asthma, smooth muscle could be identified in some instances to extend into the terminal bronchioles. Though cells were not stained to specifically identify proliferation in this investigation, the presence of muscle in terminal bronchiolar airways, which are normally devoid of smooth muscle, confirms the presence of cellular hyperplasia or metaplasia/transdifferentiation and not airway smooth muscle hypertrophy. This lesion would be anticipated to limit alveolar ventilation and, to our knowledge, has not been described in human asthmatics. Additional investigations of smooth muscle in small airways are necessary to substantiate the importance of structural changes in airway smooth muscle of small airways as a factor that can differentiate severe asthma from the less severe types. However, it is important to recognize that asthma is a multifaceted disease with heterogeneous phenotypes. Therefore, more than

one histopathologic lesion could conceivably be correlated to asthma severity in different subtypes of the disease.

Collagen deposition in the airways is a component of airway remodeling in asthma.²⁸ Airway fibrosis plays a role in the pathophysiology of incompletely reversible airway obstruction that occurs in some asthmatics.²⁸ Subepithelial fibrosis has been associated with asthma severity, frequency, duration of symptoms, and the degree of airway hyper-reactivity^{29,49-51}. However, the magnitude of collagen proliferation found in large airways does not differentiate severe asthma from milder forms of this condition.⁴⁶⁵² Furness and colleagues have investigated pulmonary lesions associated with the barn-associated form of RAO and have suggested that collagen deposition may not be a feature of RAO. Their results demonstrated that noncartilaginous airways of affected horses did not present differences in the amount of collagen compared with airways from clinically normal horses.⁵³ Our study, by contrast, demonstrates that, relative to non-diseased control horses, horses with SPARAO have a significantly greater frequency of peribronchiolar fibrosis. This finding is in line with that of airway remodeling that is described in association with chronic asthma and may have been facilitated by the utilization of pentachrome staining that clearly stains collagen yellow.

Airway occlusion by mucus and/or inflammatory cells occurs commonly in horses with barn-associated RAO, where a strong correlation between disease exacerbation and mucus hypersecretion has been identified in SPARAO.⁴³ Occlusion occurs even during remission when inflammation, clinical signs and bronchospasm are reduced or nonexistent.⁵⁴ In this investigation the odds of a horse with SPARAO having airway occlusion by mucus and/or inflammatory cells was 4.2 times greater than a horse without

SPARAO. This finding is consistent with that of Costa whose investigation identified mucus accumulation in small airways as a predominant histopathologic alteration in the lungs of SPARAO-affected horses.¹¹ Similar to that study, our quantitative approach clearly demonstrates that airway occlusion by mucus and/or inflammatory cells is significantly increased in horses with SPARAO. Horses with SPARAO were 4.2 times more likely to have airway occlusion by mucus and inflammatory cells than were non-diseased control horses. Frequency of airway occlusion by mucus and/or inflammatory cells was not significantly different in horses under 15 years and over 15 years of age, with an odds ratio of 2.5 and confidence interval that included unity, indicating that age was not a factor in the frequency of this lesion. Accordingly, airway occlusion by mucus and inflammation is conceivably a major contributor to clinical signs during disease exacerbation. In human asthma, mucus plugs combined with an admixture of inflammatory cells have been reported in cases of sudden asthma death.⁵⁵ Also, earlier investigations have demonstrated a 31 fold increase in mucus accumulation in terminal airways of patients who died of severe acute asthma attack.²⁵ Our finding of significant airway obstruction by mucus and inflammatory cells in SPARAO is consistent with the previously indicated relevance of airway occlusion in inducing the clinical signs observed in severe asthma.

Goblet cell hyperplasia/metaplasia is acknowledged as a key component of airway remodeling in asthma.^{26,29,45,46} During our study we recognized some confusion in the literature with regards to the use of the term metaplasia and hyperplasia. Not infrequently, metaplasia has been referred to as hyperplasia. Hyperplasia refers to proliferation that yields the same parental cell type, while metaplasia refers to cellular

differentiation to an alternate cell type. By contrasting the distribution of goblet cells in horses with RAO and normal horses using morphometric investigation, Range et al. determined that metaplasia predominates in the bronchioles of horses with barn-associated RAO, whereas goblet cell hyperplasia is evident in bronchi.⁵⁶ Goblet cell hyperplasia/metaplasia contributes to airway obstruction via increased cell size and increased production of mucus. In this investigation, goblet cell hyperplasia/metaplasia constituted a highly significant difference between SPARAO horses and controls. Horses with SPARAO were 37.6 times more likely to have goblet cell hyperplasia/metaplasia than were control horses. Frequency of goblet cell hyperplasia and metaplasia was not significantly different between age groups with an odds ratio of 0.63 and confidence interval that included unity, indicating that age was not a factor in the frequency of this lesion. Therefore, goblet cell hyperplasia/metaplasia is a lesion whose increasing frequency is highly significantly associated with SPARAO. In fact, the frequency of goblet cell hyperplasia/metaplasia tended to decrease with age making this lesion helpful in differentiating the histopathologic diagnosis of SPARAO from other causes of respiratory impairment, particularly in horses over 15 years of age.

Analyses of BALF collected from SPARAO-affected horses have demonstrated that the inflammatory exudate consists primarily of neutrophils and lymphocytes and the presence of eosinophils is less common.¹¹ In addition, cough frequency has been strongly correlated to the magnitude of neutrophil counts in BALF of barn-associated RAO-affected horses.⁴³ This investigation demonstrated that airway adventitial inflammation, which was predominantly neutrophilic in nature, is a histopathologic feature that is significantly increased in horses with SPARAO. Horses with SPARAO were 3.0 times

more likely to have airway adventitial inflammation than were non-diseased control horses. The frequency of airway adventitial inflammation was not significantly different in horses under 15 years and over 15 years of age, with an odds ratio of 1.27 and confidence interval that included unity, indicating that age was not a factor in the frequency of this lesion. Our findings are very similar to previous research involving severe human asthma that is also characterized by neutrophilic lung inflammation. In fatal asthma attacks, bronchiolar neutrophilic inflammation predominates and eosinophilic inflammation is less prevalent. This finding has led to the hypothesis that fatal attacks of asthma may be triggered by stimuli that elicit neutrophilic inflammatory responses.⁵⁷ Neutrophils have also been recognized as the major inflammatory cell present in acute severe asthma that necessitates mechanical ventilation.⁵⁸ Small airway inflammation is highly correlated with severe asthma attacks. Previous research has demonstrated that, in severe cases of asthma, the most accentuated inflammatory process occurs in the noncartilaginous airways, contributing to the predisposition of small airways to be the major location of obstruction.^{35, 59} The common finding of neutrophilic small airway inflammation observed in SPARAO, barn-associated RAO, and in human severe asthma, suggest that these spontaneous equine diseases may be useful to investigate facets of neutrophilic inflammation in severe human asthma.

In the review of several hundred sections for this work, abnormalities of the terminal bronchioles characterized by varying degrees of extension of smooth muscle, collagen and elastic fibers into surrounding alveolar ducts, alveoli and alveolar septal walls and accompanied by varying degrees of terminal bronchiolar epithelial hyperplasia were evident. This combination of changes at the level of the terminal bronchiole has to

our knowledge not been previously described in the literature. We postulate, based upon the associated compromise of the terminal bronchiole which is the gateway to the alveolus, that terminal bronchiolar remodeling impairs alveolar ventilation in a fashion that is proportional to the severity of the lesion. Relative to non-diseased control horses, horses with SPARAO have a greater frequency of terminal bronchiolar remodeling that is highly significant. Horses with SPARAO were 3.7 times more likely to have terminal bronchiolar remodeling in their small airways than control horses. Age was also a highly significant factor that impacted the frequency of terminal bronchiolar remodeling in a manner that was independent of disease status. Horses greater than 15 years of age were 2.4 times more likely to have terminal bronchiolar remodeling than horses less than 15 years of age. This data indicates that terminal bronchiolar remodeling is a feature of the summer pasture associated form of RAO. However, frequency of this lesion was also increased in horses over 15 years of age, independent of disease status. Accordingly, from a clinical standpoint, the presence of terminal bronchiolar remodeling in horses over 15 years of age does not exclusively implicate SPARAO as a disease process.

The finding of increased elastin fibers in the peribronchiolar region of horses with SPARAO is novel. This lesion has also not been previously reported in barn-associated RAO. This observation was fortuitous because of the use of pentachrome staining that clearly stains elastin fibers. Peribronchiolar elastin fiber frequency was increased in horses with SPARAO and this increase was highly significant in relation to normal control horses. Horses with SPARAO were 4.2 times more likely to have increased peribronchiolar elastin fibers in their small airways than were control horses. Age was also a highly significant factor that strongly positively impacted the frequency of

peribronchiolar elastin fibers in a manner that was independent of disease status. Horses greater than 15 years of age were 2.9 times more likely to have peribronchiolar elastin fibers than horses less than 15 years of age. This data indicates that peribronchiolar elastin fibers are a feature of the summer pasture associated form of RAO. However, the frequency of the lesion in horses over 15 years of age makes it difficult to exclusively implicate SPARAO as the etiology when increased elastin fibers are detected in the small airways of horses over 15 years of age. However, in such cases clinical characteristics of the disease would also be collectively interpreted to clarify the relevance of SPARAO as a diagnosis.

In conclusion, goblet cell hyperplasia/metaplasia and increased smooth muscle with evidence of hyperplasia are two lesions whose frequency is increased in a highly significant manner in horses with SPRAO and for which there is no significant increase in lesion frequency in horses over 15 years of age. Airway adventitial inflammation and airway occlusion by mucus and inflammation demonstrate a statistically significant increase in frequency and there is also no significant increase in frequency of these lesions with increasing age. These 4 lesions: goblet cell hyperplasia/metaplasia, increased airway smooth muscle, airway adventitial inflammation and airway occlusion by mucus and inflammatory cells, provide a panel of histopathologic findings whose collective presence is useful in refining the histopathologic diagnosis of SPARAO.

Table 4.1 Criteria for histologic scoring of lung from horses with summer pasture associated recurrent airway obstruction

<p>1-Smooth muscle hypertrophy and hyperplasia</p> <p>0 = generally one identifiable layer that is predominantly discontinuous with no irregular branching</p> <p>1 = mildly thickened smooth muscle bundles generally 2-3 cells thick with longer expanses of smooth muscle continuity</p> <p>2 = moderately thickened smooth muscle bundles generally 3-5 cells thick with short extensions into surrounding alveolar walls</p> <p>3 = severely thickened smooth muscle bundles greater than five cells thick with longer segments and complex extensions into surrounding alveolar walls</p>	<p>2-Peribronchiolar fibrosis with collagen</p> <p>0 = minimal loose connective tissue in adventitia</p> <p>1 = mild</p> <p>2 = moderate</p> <p>3 = severe</p>	<p>3-Peribronchiolar elastin fibers</p> <p>0 = minimal scattered elastic fibers in adventitia</p> <p>1 = mild</p> <p>2 = moderate</p> <p>3 = severe</p>	<p>4- Airway occlusion by mucus or inflammatory cells</p> <p>0 = absence of mucus and inflammatory cells</p> <p>1 = small amounts of mucus in bronchiolar lumen</p> <p>2 = moderate mucus in bronchiolar lumen with up to 50% filling of luminal diameter</p> <p>3 = severe mucus in bronchiolar lumen with extension into alveolar ducts and surrounding alveoli and greater than 50% bronchiolar occlusion</p>
<p>5-Airway adventitial inflammation</p> <p>0 = absence of adventitial inflammation or occasional rare lymphocytes</p> <p>1 = mild infiltration of bronchiolar inflammatory cells limited to adventitia</p> <p>2 = moderate infiltration of bronchiolar adventitia resulting in moderate thickening and mild transepithelial inflammatory cell migration</p> <p>3 = severe infiltration of adventitia resulting in marked thickening with extension to lamina propria and transepithelial migration as well as extension into peribronchiolar alveoli</p>	<p>6-Terminal bronchiolar remodeling</p> <p>0 = open terminal bronchiolar junction and abrupt transition to alveolar duct</p> <p>1 = mild terminal bronchiolar branching with mild discontinuous extension of smooth muscle, collagen and elastic fibers into surrounding alveolar ducts and alveoli</p> <p>2 = moderate terminal bronchiolar branching with moderate extension of smooth muscle, collagen and elastic fibers in longer segments into surrounding alveolar ducts and alveoli with some terminal bronchiolar hyperplasia</p> <p>3 = complex extensions of smooth muscle, collagen and elastic fibers generally continuous in a radiating pattern into surrounding alveolar septal walls accompanied by terminal bronchiolar epithelial hyperplasia</p>	<p>7-Goblet cell hyperplasia or metaplasia</p> <p>0 = no identifiable goblet cells in terminal bronchioles</p> <p>1 = few to 10% of epithelial cells</p> <p>2 = 10-30% of epithelial cells</p> <p>3 = >30% of epithelial cells</p>	

Table 4.2 Statistical analysis of the frequency of 7 histologic lesions based upon disease status and age

Histological Parameter	Fixed Effects	Odds Ratio	P-Value
Smooth muscle hypertrophy and hyperplasia	Status	2.515	0.0009
	Age Group	1.536	0.0014
Peribronchiolar fibrosis with collagen	Status	3.750	0.0107
	Age Group	2.724	0.0437
Airway occlusion by mucus or inflammatory cells	Status	4.179	0.0369
	Age Group	2.504	0.1690
Airway adventitial inflammation	Status	3.008	0.0127
	Age Group	1.274	0.5739
Terminal Bronchiolar Remodeling	Status	3.686	<.0001
	Age Group	2.394	0.0004
Goblet Cells Hyperplasia or Metaplasia	Status	37.622	<.0001
	Age Group	0.628	0.0629
Peribronchiolar Elastin Fibers	Status	4.237	0.0002
	Age Group	2.881	0.0043

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APPENDIX A
CHEMICAL SOLUTIONS USED IN THE MODIFIED MOVAT'S
PENTACHROME I STAIN

Chemical Solutions Used in the Modified Movat's Pentachrome I Stain

- Stock solution A

Orcein - 0.5 g,

Hydrochloric acid, concentrated - 0.5 ml

Ethanol, 70% - 250 ml

- Stock Solution B

Hematoxylin - 4 g

Ethanol, 100% - 80 ml

- Stock Solution C

Ferric Chloride – 4.8 g

Distilled water – 45 ml

- Stock Solution D

Iodine – 0.5 g

Potassium Iodine – 1g

Distilled water – 48 ml

- Working Elastic Solution

Stock Solution A – 75 ml

Stock Solution B – 24 ml

Stock Solution C – 15 ml

Stock Solution D – 15 ml

- Mucin Stain

Alcian Blue – 1 g

Acetic Acid, Glacial – 1 ml

Distilled water – 99 ml

- Ammoniated Ethanol

Strong ammonia – 5 ml

Ethanol, 95% – 95 ml

- Plasma stain

Crocein scarlet 1% aqueous – 4 ml

Acid fuchsin 1% aqueous – 1 ml

Acetic acid, 0.5% - 95 ml

- Polyacid

Phosphotungstic acid – 5 g

Distilled water – 100 ml

- Fiber Stain

Spanish saffron – 3g

Ethanol, 100% - 48 ml

Close the container tightly and keep this suspension in a 60°C oven for two days. Then pour the liquid out and store it in a dark sealed container to prevent hydration.