Comparison of Airway Response in Recurrent Airway Obstruction-Affected Horses Fed Steamed versus Non-steamed Hay

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Biomedical and Veterinary Sciences

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June 21, 2012
Blacksburg, Virginia, USA

Keywords: Horses, Recurrent Airway Obstruction, Hay, Steaming, Fungus

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Recurrent Airway Obstruction (RAO)-affected horses experience bronchoconstriction and airway inflammation in response to inhalation of irritants including hay molds. Steaming hay reduces fungal content, but the effect on the antigenic potential has not been investigated. We tested the hypothesis that RAO-affected horses develop less severe clinical disease when fed steamed versus non-steamed hay and this reduction coincides with decreased hay fungal content.

Six RAO-affected horses in clinical remission were divided in two groups and fed steamed or non-steamed hay for 10 days using a two-way cross-over design. Hay was steamed using a commercial hay-steamer. Clinical assessment was performed daily. Full assessment, including airway endoscopy, tracheal mucous scores and maximal change in pleural pressure, was performed on days 1, 5, and 10. Bronchial fluid sampling and cytology were performed on days 1 and 10. Hay core samples were collected pre- and post-steaming and cultured to determine fungal and bacterial concentrations.

Statistical analysis was based on data distribution and quantity and performed using SAS®. P-value <0.05 was significant.

Steaming significantly decreased the number of bacterial and fungal colony-forming-units in hay. Horses fed non-steamed hay experienced a significant increase in clinical score and a trend towards airway neutrophilia, while parameters were unchanged in horses fed steamed hay. Only horses fed non-steamed hay experienced a significant increase in tracheal mucous score. Horses fed steamed hay gained significantly more weight compared to horses fed non-steamed hay, even though the amount of hay consumed not greater on a dry matter basis.

These results indicate that steaming reduces the RAO-affected horse's response to hay which coincides with a reduction in viable fungal content of hay.

ACKNOWLEDGEMENTS

I would like to thanks to my advisor, Dr. Virginia Buechner-Maxwell and my committee members, Dr. Kent Scarratt, Dr. Katherine Wilson, Dr. Carolina Ricco and Dr. Jennifer Hodgson for their help, advice and patience.

I would also like to thank Dr. Steven Werre for his help and advice in the statistical analyses of the data.

I would like to thank all the technicians of the Veterinary Teaching Hospital of the Virginia-Maryland Regional College of Veterinary Medicine and volunteers from Radford University and Virginia Tech for their help in patient care and data collection.

Finally, I would like to thank the owners of the horses used in my study.

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CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION

Equine recurrent airway obstruction (RAO), also known as chronic obstructive pulmonary disease (COPD) or heaves, is a common, chronic and incurable respiratory condition that affects the adult horse.[1] It resembles human asthma and may be associated with pulmonary hypersensitivity to inhaled allergens. The mean age of horses diagnosed with RAO is 7 years of age and clinical signs, which vary between affected horses, include coughing, exercise intolerance, and increased respiratory effort. [2] Diagnosis is often based on history and clinical signs alone; however, additional tests including cytological evaluation of airway secretions collected by bronchoalveolar lavage (BAL) assist in confirming the diagnosis.[2] The discovery of an neutrophilic leukocytosis on cytological examination of fluid from the lower airway [3] in the absence of an infectious cause is the typical finding associated with active RAO.

The pathogenesis of RAO is multifactorial and not completely understood. RAO is an allergic inflammatory response to inhaled allergens including inhaled dust, mold, and endotoxin. [2] Horses are commonly exposed to these allergens in the stable air, hay and bedding. In normal horses, exposure to these allergens causes lower airway inflammation, while exposure in RAO-affected horses causes lower airway inflammation, bronchoconstriction, bronchospasm, hyperresponsiveness and mucous secretion. A familial predisposition to RAO has been shown and genetic factors play a role in the severity of clinical signs.[4, 5]

The most important aspect of treatment and prevention of clinical disease in RAO-affected horses is environmental management to reduce exposure to allergens. Medications, including corticosteroids and bronchodilators, are often used in acute episodes to help induce remission; however, potential side effects make long-term administration of medications undesirable.

Potential long term effects of RAO include lost days in work, cardiac and skeletal muscle pathology such as cor pulmonale and muscle wasting, [6, 7] and increased susceptibility to respiratory pathogens due to decreased mucociliary clearance. In addition, the increased work of breathing increases energy demands and results in lower body condition scores.[8]

2. PATHOPHYSIOLOGY – INTRINSIC FACTORS

2.1 *Genetic predisposition*

Genetics and heritability play a role in the development of recurrent airway obstruction. Foals with one RAO-affected parent have a 3-fold increase in risk of developing the disease and foals with two RAO-affected parents have a 5-fold increase.[9] The mode of inheritance is not clear as one family of horses demonstrated autosomal dominant inheritance, while another family demonstrated autosomal recessive inheritance.[10] There is also a strong genetic predisposition for moderate to severe signs of chronic lower airway disease.[4] Human asthma also appears to be inheritable and likely involves multiple genes. [11]

2.2 Inflammatory and immune response

Both innate and humoral immunity contribute to the development of RAO. Airway exposure to allergens induces an innate immune response and an increase in pro-inflammatory cytokines and chemokines that function as signaling molecules causing additional inflammation. Dendritic cells and alveolar macrophages contribute to innate immunity by antigen presentation and clearing foreign allergens and pathogens from the alveoli.[12-15]

Lymphocytes are an important immune cell type and are classified as B or T lymphocytes based on their origin. T cells can be divided into subsets that include the helper T cells and cytotoxic T cells. T helper cells are further categorized based on the cytokines that they produce and the effect that they have on the inflammatory response. T-helper 1 (Th1) cell response is

associated with a cellular immune response, macrophages and secretion of IFN-gamma and TNF-alpha. Th2 cells are associated with B cells and the humoral immune system. Human asthma is mediated by Th2 and B cells. [2]

In addition to Th2 cells, human asthma is also associated with IgE whose synthesis is stimulated by IL-4 and eosinophilic inflammation through production of IL-5, however the role of eosinophils in equine RAO is questionable.[16-18] Within horses with RAO, the cytokines and type of T cell population involved vary depending on the stage of clinical disease.[19] Increased concentrations of pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-8 produced by alveolar macrophages can be measured in RAO affected horses as compared to healthy horses.[20, 21]

Interleukin-8 (IL-8) is a chemoattractant and activator of neutrophils which is important in the development of neutrophilic airway inflammation. [22] Comparison of cytokine production in RAO-affected horses to normal horses has yielded contradictory results. [23, 24] This has led some authors to conclude that RAO is not a specific Th1 or Th2 response.[25] However, other work showing increased expression of IL-4 and IL-5 in RAO-affected horses supports the fact that Th2 is the predominate response. [21, 26, 27]

An additional interleukin (IL-17) is responsible for neutrophil chemotaxis, activation and maturation. It is downregulated in BAL fluid in healthy horses and upregulated in RAO-affected horses placed in a challenge environment. [28]

Airway neutrophilia is a rapid response to natural challenge by inhalation and the time period between exposure and BAL fluid neutrophilia can be as short as 5 hours. [29, 30] Because this response also occurs to a lesser degree in normal horses exposed to a dusty environment, this is likely a non-specific response. [31] There is also evidence of delayed airway

neutrophil apoptosis and possible differences in neutrophil activation in RAO-affected horses.[32]

2.3 Mucous secretion and accumulation

RAO-affected horses usually have an increase in tracheal mucus. This accumulation can be the result of several different mechanisms including increased volume of secretion, decreased clearance and a change in the physical properties of the mucus.[33] It is likely that all three components play a role in increasing tracheal mucous accumulation. Increased mucus production is seen in humans with asthma and is influenced by several mechanisms including activation of calcium activated chloride channel 1 (CLCA1), epidermal growth factor receptor (EGFR), mucin gene (MUC5AC), IL-13 and interferon- gamma.[34] Two mucin genes have been identified in the horse: equine mucin gene 5AC (eqMUC5AC) which is expressed in the glandular stomach, colon and airways, and equine mucin gene 2 (eqMUC2) which is expressed only in the colon. RAO-affected horses have an upregulation of eqMUC5AC and this may increase mucus production within the airway in response to exposure to aerosolized debris.[35] Goblet cell metaplasia and hypersecretion are also often present in RAO affected horses.[36] The mucus produced by clinical RAO-affected horses has increased viscoelasticity, decreased mucociliary clearability and decreased cough clearability compared to normal horses.[33] These changes contribute to the increase amount of tracheal mucus seen in RAO-affected horses.

2.4 Bronchoconstriction and bronchospasm

Bronchoconstriction is a key component in the pathology of RAO. Airway smooth muscle has a large amount of beta-2 adrenoreceptors as compared to other smooth muscle.[37] Beta-2 receptor activation results in bronchodilation, while increased cholinergic activity induces brochospasm. Horses with RAO have decreased density of these receptors in both the lung

parenchyma (33%) and bronchial tissues (42%).[37] Although their density is decreased, studies have reported contradicting results regarding the functionality of beta-2 receptors in RAO-affected horses. [38-40]

2.5 Effects of Chronic Disease

Recurrent airway obstruction leads to the development of chronic changes such as smooth muscle remodeling and pulmonary fibrosis. An increase in smooth muscle mass has been shown to be a component of airway remodeling in human and rodent asthma patients.[41] In horses with RAO, smooth muscle hyperplasia occurs resulting in increased smooth muscle mass.[42]

Alveolar fibrosis has also been described in RAO-affected horses.[34, 43] These changes may not return completely to pre-disease levels in RAO-affected horses and may result in poor response to bronchodilators. Proteases, specifically metalloproteases, are implicated as a possible cause for these changes.[34]

3. PATHOPHYSIOLOGY – EXTRINSIC FACTORS

The horse's environment plays a vital role in the pathogenesis of RAO and many of the prevention and treatment strategies are aimed at modifying the horse's environment. Several studies have shown an association between environment and development of clinical signs of RAO.[12, 44-48] While on pasture, normal and RAO-affected horses demonstrate similar clinical parameters and indicators of pulmonary function. When moved to a stall and fed a diet low in aeroallergens, RAO-affected horses in clinical remission showed airway reactivity greater than normal horses, but less than RAO horses during an acute episode.[44, 48] Additional work shows an improvement in lung function of RAO-affected horses with acute, severe disease within 6 hours after modification of the environment. These horses also return to clinical

remission when placed on pasture.[12, 46, 47, 49] Aspects of the environment that need to be assessed include air quality, ventilation, hay, mold, endotoxin and bedding.

3.1 *Air quality and ventilation*

The quality of air in a stable affects both the incidence and severity of equine airway disease.[45, 50] Good quality air is determined by the number of air changes per hour and in lower dust environments, four changes per hour is considered satisfactory. Particle size plays a large role in determining the final location of the particles in the airway. Larger particles rarely reach the lower airway, while particles less than five microns in diameter will reach the lower airway and can induce airway inflammation.[51] Types of particles that are less than five microns in diameter include mold spores and dust and both are implicated in the development of clinical disease associated with RAO.[51] The process of cleaning a stall doubles the dust concentration in a barn with good ventilation and increases the concentration up to 16.8 fold in barns with poor ventilation.[52]

3.2 *Hay*

Feeding hay is commonly associated with the development of RAO.[53] When RAO-affected horses are exposed to a hay dust challenge, they develop the typical clinical signs of RAO including airway inflammation, as evidenced by a neutrophilia and mucus accumulation. Normal horses exposed to the same dust challenge experience airway neutrophilia, but to a lesser degree than RAO-affected horses and they do not show other typical signs of RAO.[54] In addition to dust particles, hay also contains varying amounts of mold with increased mold content seen in hays with higher moisture content.[55] Mold species in hay may include *Aspergillus fumigates, Faenia rectivirgula* and *Thermacintomyces vulgaris*. The method of

feeding hay affects the amount of dust particles a horse may inhale. [56] Soaking hay can decrease the amount of respirable dust.[56]

3.3 *Mold*

Hay molds including *Faenia rectivirgula* (*Micropolyspora faeni*), *Aspergillus fumigatus*, and *Thermacintomyces vulgaris* have been found to be important in the pathogenesis of RAO.[57, 58] When RAO-affected horses were exposed to *A. fumigatus*, total cell count in BAL fluid and cytokine production (TNF-alpha, IL-1beta, and IL-8) were increased.[59] Exposure to aerosolized *A. fumigatus* extract also increased the percentage of neutrophils in BAL fluid and higher exposure levels induced lung dysfunction.[60] *F. rectivirgula* exposure caused an increase in total cell count and neutrophil number in BAL fluid, but did not affect airway responsiveness.[61] Exacerbation of RAO is also associated with exposure to other fungal spores including *Basidospores spp.*, *Nigrospora spp.* and *Curvularia spp*.[62]

3.4 *Endotoxin*

Endotoxin is implicated in the development of RAO as it stimulates the innate immune response. Endotoxin increases expression of inflammatory mediators, including TNF-alpha, and results in neutrophil activation, margination and transmigration. Exposure to endotoxin increases airway neutrophilia in normal and RAO-affected horses in a dose dependent manner yet endotoxin alone does not increase clinical scores, airway reactivity or mucus accumulation in either group.[63, 64] Exposure to *A. fumigatus* extract without endotoxin yielded lower airway inflammation when compared to extract with the endotoxin. Based on these findings, endotoxin appears to amplify the airway response to other irritants, but does not induce severe clinical signs of RAO without other allergens.

3.5 *Bedding*

Common bedding choices for horses include straw and wood shavings. Less common beddings include peat moss, cardboard and paper. The type of bedding chosen can influence the amount of dust and mold within the environment.[65] Wood shavings may have up to 31,500 particles of respirable dust per liter compared to 11,600 particles per liter for good straw and 5,700 particles per liter for cardboard.[44] Wood shaving are more resistant to mold growth compared to plant-based material including paper.[45] Dust concentrations during stall cleaning may reach 10-15 mg/m^3 which is greatly elevated above air hygiene standards of <2.5 mg/m^3. [66]

4. DIAGNOSIS

It is possible to make a preliminary clinical diagnosis of RAO based on the horse's history and clinical signs.[2] However, additional evaluation may be required to confirm the clinical impression, especially in mildly affected horses. Previous studies indicate that clinical examination has a low to moderate sensitivity and predictive values for diagnosing RAO. [67] Clinical signs reported by the owner during an episode of RAO may include coughing, serous nasal discharge, increased expiratory effort, exercise intolerance and nostril flare.

A complete physical examination should be performed on all cases of suspected RAO and to eliminate additional causes of respiratory disease. Clinical findings that support a diagnosis of RAO include diffuse pulmonary wheezes and crackles peripherally on thoracic auscultation that usually occur during expiration. These changes are the result of forceful air movement through a narrowed airway and the accumulation of inflammatory debris in the small airways. Percussion of the thoracic wall can be used to determine the location of the lung field and a caudal shift of the caudal lung border is often seen in horses with RAO.[68] Hypertrophy

of the external abdominal oblique muscles resulting in a “heave line” is often present in RAO horses due to increased work required during exhalation.[2]

In an effort to provide a more client-friendly assessment of the clinical signs of RAO, a clinical scoring system has been described as a subjective assessment of severity of clinical signs.[69] A score of 1 to 4 is assigned for abdominal effort and nostril flare and the scores are combined for a total clinical score. Total clinical scores greater than 5 are associated with a significant difference in peak expiratory and inspiratory flow, dynamic elastance, and maximal change in pleural pressure.[69] Below a score of 5, there are fewer significant differences in pulmonary function, but considerable airway obstruction is present, suggesting that airway disease is underdiagnosed when using only clinical signs. [69]

Table 1: Nostril Flare and Abdominal Push Score (NAPS) Clinical Scoring System

	Attribution criteria
Abdominal expiratory effort	
1	No abdominal component to breathing
2	Slight abdominal component
3	Moderate abdominal component
4	Severe, marked abdominal component
Nostril flaring	
1	No flaring
2	Slight, occasional flaring of nostrils
3	Moderate nostril flaring
4	Severe continuous flaring during each respiration

Total scores and Clinical Signs of RAO	
2	No signs
3-4	Mild signs
5-6	Moderate signs
7-8	Severe signs

Laboratory blood work findings are often unremarkable in horses affected with heaves, but can be helpful in ruling out other diseases. Mild and inconsistent increases in packed cell volume and elevated mean erythrocyte corpuscular volume may be seen in chronic respiratory cases.[70] White cell counts and immunoglobulin to albumin ratios in peripheral blood in RAO horses are unaffected by barn exposure or return to pasture. [47] Arterial blood gas analysis may show evidence of hypoxemia with normocapnia or hypercapnia in severe cases with compromised lung function.[71]

Diagnostic imaging options, including radiology and ultrasonography, are most useful in ruling out other respiratory conditions. Thoracic radiographs are often unremarkable in mild RAO cases with changes only seen in advanced RAO cases. Clinically affected horses may show an increased broncho-intersitial pattern diffusely throughout the lung field with evidence of small airway thickening. Hyperinflation may be present causing flattening of the diaphragm and a more lucent lung field. Ultrasonography allows the examiner to reliably detect the caudal lung borders but its use is limited by its inability to penetrate through aerated lung.[68] Changes in the pleural surfaces can be detected in RAO-affected horses. In addition, pleural surface irregularities are not pathognomonic for RAO. These limitations make ultrasound an insensitive and unspecific indicator of RAO. [72]

Upper airway endoscopy is used to evaluate tracheal mucus accumulation and carina thickening. A proposed mucous scoring system correlates an increase in tracheal mucous score with increased severity of clinical disease as evidenced by increased neutrophil accumulation and decreased mucociliary clearance.[73]

Table 2: Tracheal Mucous Scoring System

Mucus Grade	Description
0	No visible mucus
1	Singular small blobs
2	Multiple blobs only partially confluent
3	Mucus ventrally confluent
4	Large ventral pool
5	Profuse amounts of mucus occupying more than 25% of the tracheal lumen

Trachea carina thickening is not correlated with inflammation in normal or RAO horses. Tracheal septum thickness does not correlate with clinical, endoscopic or cytological assessments of lower airway inflammation.[74] Horses 10 years of age and older have significantly thicker tracheal septum scores compared to younger horses; however, there is no

difference in tracheal septum scores between normal and RAO horses while clinical or in remission.[74]

Cytological examination of respiratory tract secretions has been reported as a diagnostic tool for RAO.[2] Techniques for transtracheal washes and broncho-alveolar lavage (BAL) have been previously described.[75] Bronchoalveolar lavage is usually indicated as it retrieves fluid from the small airways, specifically the bronchioles and alveoli, and provides information regarding the current inflammation within the lower airway. BAL fluid cytology correlates well with clinical signs and the pathophysiologic processes of RAO. [47] Transtracheal wash fluid tends to be more variable than BAL fluid, although a neutrophilia is often present.[76] Serial BALs at 5 to 7 week intervals have been shown to be safe in dogs and can be used to assess response to therapy. [77] Serial BALs in horses at four day intervals did not influence cytological analysis of the fluid.[78] The procedure involved in collection BALs significantly decreases pulmonary resistance for up to 6 hours and increases lung elastance. [79] Intravenous administration of butorphanol and intratracheal administration of lidocaine (0.66%) reduces coughing during BAL and does not alter the amount of BAL fluid retrieved or its cytologic composition.[80] Other antitussive medications failed to reduce the intensity and frequency of coughing. [80]

BAL is performed using a flexible cuffed nasobronchial tube passed blindly into the trachea and advanced until it becomes wedged in a bronchus.[81] The cuff is inflated to minimize fluid being recovered from the upper airway. Sterile saline (usually between 100 and 500 mls) is infused through and then retrieved through the tube for fluid analysis. Samples collected have been shown to be representative of the entire lung in horses that have generalized (diffuse) lung disease.[82]

When collecting a BAL fluid sample for cytological analysis, the sample should be maintained on ice until it is processed. Processing should be performed shortly after the collection. Cell morphology can become deteriorate after 8 hours when stored at 38 degrees Celsius, 24 hours at 18 degrees Celsius and 48 hours at 4 degrees Celsius.[83] Cell viability is influenced by the time period prior to fixing as well as storage temperature.[83] All aliquots from a single BAL are considered to be representative of the lavaged lung segment. [84] Because the fluid obtained is often dilute, a cytospin preparation is often used for cytological evaluation. In addition, cytospin preparations preserve the cell morphology better than direct smear. However, assessment of a direct smear does allow a reliable diagnosis of RAO. [85]

Cytology of BAL fluid in horses is used to determine the severity of lower airway inflammation. BAL samples of normal horses are composed mostly of macrophages (40-70%) and lymphocytes (30-60%).[75] Normal neutrophil percentage is less than 5% and horses with RAO usually have greater than 25% neutrophils.[86, 87] Percentages within the range of 5% to 25% do not exclude RAO as a diagnosis and further evaluation of the horse would be warranted. An increased percentage of neutrophils in the absence of evidence of infection is highly sensitive (100%) and moderately specific (64%) for the diagnosis of RAO.[67] The percentage of neutrophils found in the BAL fluid of RAO-affected horses has been shown to increase with the severity of the condition and on the level of airway allergen exposure.[88] [54, 89, 90] However, the results of BAL cytology should be interpreted within the context of the clinical picture, as Holcomb and colleagues showed that neutrophil percentage can increase in normal young and old horses based on housing conditions and this increased percentage is not associated with clinical RAO.[91] The mean relative neutrophil count in BAL fluid from clinically affected RAO horses is significantly greater than healthy horses. [92]

A significant difference is not present between healthy horses and RAO-affected horses during remission in total cell count or relative percentages of small lymphocytes (30-60%), large mononuclear cells (40-70%), eosinophils (< 0.5%) or mast cells (<2%). [92] Airway eosinophilia is not commonly seen in horses with heaves. However, some young horses with inflammatory airway disease demonstrate airway eosinophilia and this condition may represent an early manifestation of RAO. [93] Mast cells are not often reported in BAL cytology from in RAO horses, although equine mast cells are not well visualized by most commonly used staining techniques. The role of mast cells is also not clear since normal and RAO affected horses have mast cell degranulation and release of tryptase in response to challenge with organic dust.[94] Bacterial culture of respiratory tract secretions are negative but, positive culture results can be obtained when bacteria colonize mucus secondarily to delayed mucociliary clearance.

Additional diagnostic options which are rarely used in clinical practice include scintography, lung biopsy, and pulmonary function testing. Scintigraphy has been shown to be a sensitive indicator of lung damage. Scintigraphy allows the examiner to assess alveolar clearance rate, evaluate lung ventilation, assess mucociliary clearance, evaluate the deposition of aerosolized medications within the lung, and determine inflammatory cell involvement.[95-97] Percutaneous lung biopsy by a biopsy needle or wedge resection has been previously described.[98] In horses with diffuse lung disease like RAO, there is not a significant difference between samples taken in different lung regions.[99] A biopsy sample can be taken for histological evaluation to assess the severity and extent of lung pathology. Complications of lung biopsy include epistaxis, hemorrhage, infection, respiratory distress, pneumothorax and death.[100] Histopathologic findings of lung biopsies in RAO-affected horses include bronchiolar neutrophil infiltration, peribronchiolar mast cell accumulation and peribronchial

fibrosis. [71, 101] In addition, smooth muscle hyperplasia results in 2-3 times the amount of smooth muscle in RAO-affected horses than normal horses.[42, 102]

Pulmonary function testing is mainly used in research settings due to the cost of equipment, time and technical experience required. Testing requires an esophageal balloon catheter to be passed to the distal third of the esophagus and an airtight mask to be placed over the horse's muzzle. A pressure transducer measures pressure changes within the esophagus as an estimation of the intrapleural pressure to maintain airflow within the lower airways. The most commonly described term is ΔPpl_{max}, or the maximal change in pleural pressure during tidal breathing, and is an indicator of airway function. An increase in this value indicates increased work required to breathe. Normal horses have a ΔPpl_{max} value less than 10 cm water while horses during an acute episode of RAO often have values greater than 15 cm water. Horses with RAO show significant changes in clinical score, respiratory rate, peak tidal inspiratory and expiratory pressures, dynamic compliance and pulmonary resistance when exposed to a challenge environment. [103] Increasing airway obstruction in response to natural challenge with stable housing is reflected by an increase in pleural pressures and pulmonary resistance with a decrease in dynamic compliance and PaO2.[46, 78, 104, 105] Horses with RAO also exhibit a higher expiratory to inspiratory ratio. Horses with RAO demonstrate increased airway hyperresponsiveness during acute episodes; however, there is no correlation between airway reactivity and changes in pulmonary resistance and dynamic compliance.[46] Environmental modification or treatment with atropine cause improvements in pulmonary function tests; however, the short-term response to treatment with atropine underestimates the improvement in respiratory tract function that occurred when horses are maintained on pasture.[106] Pulmonary lung function testing has a low sensitivity when compared to other

techniques (sensitivity of pulmonary resistance at 22%, dynamic compliance at 33%, and maximal change in pulmonary pleural pressure at 44% compared to sensitivity of clinical score at 66.7% and BAL neutrophil percentage at 100%) .[67]

Other rarely used diagnostics include exhaled breath condensate and three additional methods of pulmonary function testing - forced expiration techniques, impulse oscillometry and flow loops. Exhaled breath condensate is evaluated for hydrogen peroxide and ascorbic acid.[107, 108] In one study, hydrogen peroxide was shown to be present in higher concentrations in horses with RAO due to airway inflammation. This increase was inversely related to ascorbic acid and positively correlated to bronchoalveoloar lavage neutrophils and tracheal wash inflammation.[109] However, another study indicated that hydrogen peroxide concentrations were not affected by environmental management changes shown to reduce clinical signs of RAO. [107] Forced expiration techniques, originally described in anesthetized horses, but more recently described in standing sedated horses, allows for evaluation of pulmonary parameters. [108] This method had a high sensitivity, specificity and predictive value for diagnosing RAO (79-100%). [67] Impulse oscillometry is a more sensitive alternative to pulmonary function testing and may be a promising method to detect subclinical changes.[110, 111] Tidal breathing flow volume loops in a healthy horse shows biphasic inspiratory and expiratory patterns with peak flows detected early in the respective phases. As the severity of airway obstruction increases, the flow loops have a peak expiratory flow early in expiration followed by a low flow rate.[112] The use of the horse (leisure, competing at events requiring less than 5 minutes of work such as dressage, or competing at events requiring greater than 5 minutes of work such as eventing) affects the ability of flow loops to diagnose varying degrees of RAO.[113]

5. ENVIRONMENTAL MODIFICATIONS

Environmental modification is a key component in the treatment of RAO and in the prevention of future episodes. Consequently, management of RAO-affected horses should be focused on decreasing allergen exposure through modification of the horse's environment.[114] Several factors and inciting causes must be addressed including housing, bedding, ventilation, and hay exposure.[115]

A low dust environment is critical in the management of RAO-affected horses as particles smaller than five micrometers will reach the lower airway and have been implicated as an inciting cause of RAO.[51] The ideal housing for most RAO affected horses is outdoors on a grassy pasture. A significant improvement in lung function is seen within three days of changing to a pasture environment from a high dust environment with airway function improving through 30 days.[116, 117] In addition, BAL fluid neutrophilia decreases with housing on pasture, although horses with summer pasture associated RAO will develop BAL fluid neutrophilia while on pasture.[116, 117]

Hay is often implicated as a potential inciting cause as it is a source of dust and mold, specifically *Aspergillus* species. The removal of hay from the horse's diet and environment combined with the feeding of a complete, pelleted feed, cubed hay or chopped forage is ideal, although this option may be impractical or unappealing to owners due to cost or inconvenience. Soaking hay prior to feeding has been shown to reduce respirable dust concentrations[56], but soaking can negatively alter the nutritional content of the hay. Steaming hay has been shown to reduce the fungal spore content from 7,775,000 per gram to 0 post-steaming based on fungal culture growth.[118, 119] It is not known if or how steaming affects the antigenic potential of the mold spores. Many common vaccines utilize killed organisms or viruses, yet they induce an

immunologic response adequate to prevent infection by the live organism or virus. Recommendations for steaming by a commercial steamer indicate adequate steaming and killing of mold at 160 degrees Fahrenheit.[120] Proteins can start to become denatured at 122 degrees Fahrenheit[121] and the change in protein conformation inducing by steaming may affect the protein's ability to induce airway inflammation.

An additional aspect of environmental modification is bedding. Decreasing moisture content in the bedding reduces fungal growth and subsequent exposure to horses.[65] There are only small differences in dust particles between straw, wood shavings, and paper bedding when the bedding was properly stored.[122] However, it has been shown that dust concentration is higher in barns using straw bedding and variable in barns using wood shavings[106] and straw has been shown to have a higher content of mold and endotoxin than good quality wood shavings.[50] Other alternatives, such as paper, cardboard and peat moss, are available but their use is often limited due to cost and lack of ease of disposal. Their dust concentration is lower but, they can still be a source of mold spores and endotoxin in poorly ventilated areas.[44, 123] Stall cleaning also plays a role in stable dust concentrations as it increases the concentration by 3-6 times. This increase is influenced by the type of bedding and straw increases dust more than paper.[50, 65]

Adequate ventilation allows for clearance of dust from the horse's environment. If dust release from hay and bedding is low, a ventilation rate of four air changes per hour is considered satisfactory. [122] Although the reduction in dust concentration levels is important in the management of RAO, clinical scores and results of pulmonary function testing may remain elevated in RAO horses compared to normal horses in low dust environments due to irreversible airway remodeling and not airway inflammation. [124]

6. MEDICATIONS

Although environmental management is the foundation of treating and managing RAO horses, medications are often needed in acute episodes and may be needed in some cases to maintain remission. The most commonly used medications are anti-inflammatory drugs (specifically corticosteroids) and bronchodilators.[2] Other less commonly used medications include xylazine, atropine, furosemide and lidocaine. Medications can be delivered systemically or by inhalation.[2]

6.1 *Corticosteroids*

Corticosteroids are anti-inflammatory drugs which predominantly act via blockade of the arachadonic acid cascade. Inhibition of phospholipase A2 results in a decrease in eicosanoid production and various leukocyte functions.[125] Potential adverse side effects observed in horses include laminitis, adrenal suppression, bacterial pneumonia, and immunosuppression. [126] Immunosuppresion at high levels may increase the incidence of secondary infection; however, it can be beneficial in decreasing the immune response to antigens.[127] Commercially available systemic corticosteroids include dexamethasone, prednisolone, prednisone, isoflupredone acetate, and triamcinolone acetonide, while inhaled corticosteroids include beclomethasone diproprionate and fluticasone propionate. [2, 128]

Dexamethasone is the most commonly used systemic corticosteroid. It has approximately 25 times more anti-inflammatory potency than cortisol.[129] Clinical improvement may be observed as early as a few hours after administration [130], although a week or longer is often needed before maximal improvement can be evaluated. Dexamethasone has a dose dependent effect on airway neutrophilia. The recommended dose range is 0.05 – 1

mg/kg orally [78, 117] or 0.04 mg/kg daily intravenously[126]. Intravenous administration induces improvement in lung function within two hours of administration with a peak effect at 4-6 hours. In addition, intravenous administration improves airway obstruction, but does not always reduce respiratory tract neutrophilia.[78, 117, 131] One study demonstrated that following 7 days of treatment at 0.1 mg/kg IV every 24 hours, pulmonary function testing (ΔPpl_{max}) results in stabled, clinically affected horses were similar to horses on pasture and airway neutrophilia was decreased.[117, 132] However, after discontinuing the medication, airway inflammation returned to pre-treatment values in RAO-affected horses remaining in a challenge environment.[114] Oral administration of the injectable solution of dexamethasone improves airway function for up to 30 hours when used at higher doses, but is inconsistent at lower doses.[130] Additionally, oral administration of dexamethasone at 0.164 mg/kg prior to feeding in clinical RAO-affected horses shows an improvement in lung function within 6 hours of administration with a peak effect at 24 hours with a duration of effect of 30 hours. Dexamethasone has a more beneficial effect on BAL cytology than prednisolone. [133] A side effect is adrenal suppression that occurs within 2 days of treatment but resolves once the medication is discontinued.[114]

Prednisolone is less potent than dexamethasone, but has fewer reported side effects, making it an appropriate choice for mildly affected horses or to maintain remission of RAO signs. Prednisolone is rapidly absorbed with a peak serum concentration within 45 minutes of administration. [134] A rapid reduction in airway inflammation with a slower improvement in airway function has been seen in horses treated with prednisolone. [135] A potential, but rare, complication associated with long term treatment is the development of bacterial pneumonia.[136]

Prednisone is poorly absorbed when administered orally in horses and it has little to no conversion to its active form, prednisolone. [117, 134, 137]. Due to these properties, prednisone has been shown to not improve clinical signs, pulmonary function or BAL fluid cytology in RAO-affected horses and is therefore considered a poor choice to treat RAO-affected horses. [135, 137]

Triamcinolone (0.09 mg/kg IM) improves respiratory function for up to 3 weeks while horses were maintained in dusty conditions; however, adrenal function is altered for up to 6 weeks.[138] The duration of action increases the chance of side effects such as laminitis and triamcinolone is not commonly used in the treatment of RAO. To decrease complications, a second dose should not be given within 6 weeks of the initial injection.

Isoflupredone acetate is a corticosteroid with glucocorticoid and mineralocorticoid properties. It is as effective as dexamethasone in the treatment of RAO, but is associated with hypokalemia. [126] Adrenal suppression is longer with isoflupredone acetate than with dexamethasone.[126]

Inhaled corticosteroids include beclomethasone dipropionate and fluticasone proprionate. They may be administered by a metered dose inhaler with a mask.[139] These drugs have fewer systemic side effects; however, they are more expensive than systemic corticosteroids making them less desirable for long term treatment. Their use has been shown to be safe and effective in horses. [21, 116] Beclomethasone dipropionate reduces the clinical signs of RAO and improves the results of pulmonary function testing within 24 hours of treatment. [132] The response is less than parenterally administered dexamethasone. Lower dosage administration does not have an effect on BAL fluid cytology. [140] This medication does have systemic absorption when administered by inhalation as shown by decreased serum cortisol concentrations. [114] After 2

days of treatment, respiratory effort is improved and after 10 days, neutrophil counts in BAL fluid is decreased. [114] Without a concurrent change in environment, neutrophil counts will return to pre-treatment levels once the medication is discontinued. Adrenal gland suppression occurs in a dose dependent manner but resolves rapidly after discontinuing the medication. Administration of fluticasone proprionate has also been shown to decrease clinical signs, improve pulmonary function and decrease BAL neutrophil counts in RAO-affected horses. [21] [128] When treatment is initiated early, recovery time may be reduced with treatment in severely affected horses. [141]

6.2 *Bronchodilators*

Bronchodilators used in veterinary medicine target airway smooth muscle and include anticholinergic agents, beta-2 adrenergic agonists and methylxanthines.[142] These medications can be given by inhalation, orally or intravenously.

Beta-2 adrenergic agonists include clenbuterol, albuterol, pirbuterol acetate, salmeterol and trimetoquinol. Clenbuterol is a commonly used beta-2 adrenergic agonist that can be administered orally or intravenously. Side effects can include nervousness, anxiety, sweating and trembling at high doses. [143] Up to 25% of horses do not respond to therapy.[143] Therapeutic effects are decreased after 2 weeks of treatment. It has bronchodilatory, anti-inflammatory [144, 145] and improved mucociliary clearance effects [146]. Clenbuterol reduces the expression of pro-inflammatory cytokines and chemokines in alveolar macrophages and increases the expression of interleukin-6, which has both pro- and anti-inflammatory properties. [145]

Albuterol is a commonly used aerosolized bronchodilator with a rapid onset of action (within 5 minutes); however the duration of action is fairly short (up to 3 hours).[147] No adverse effects are seen at commonly used dosages. [147]

Pirbuterol acetate, trimetoquinol and salmeterol are less commonly used in clinical practice. Pirbuterol acetate induces improvements in pulmonary function testing parameters for up to one hour post-treatment.[148] Trimetoquinol is more commonly used in human asthma patients. The onset of action is very rapid and the duration of action is short in horses.[149] When given intravenously, the medication has potent cardiovascular stimulatory effects; however these same negative side effects are not seen when given by aerosolization and it is therefore, considered a safe drug for aerosol use. [150] Salmeterol induces a reduction in pulmonary resistance and pleural pressure for 6 hours. [151]

The methylxanthine medications include theophylline and aminophylline. They are phosphodiesterase inhibitors that cause bronchodilation by decreasing cAMP breakdown and relaxing smooth muscle. [142] Theophylline has not been shown to improve lung function in RAO affected horses. [152] Aminophylline alleviated clinical signs in 50% of cases in one study.[153] The margin of safety with these two medications is low.

Phosphodiesterase inhibitors inhibit TNF-alpha and inhibit equine neutrophil function in vitro , making them beneficial for their anti-inflammatory properties. The most commonly used phosphodiesterase inhibitor used in equine medicine in pentoxyphylline. Pentoxyphylline is overall poorly absorbed in horses, although there is great variation between horses. [154] This medication reduces pulmonary resistance but does not affect BAL fluid cytology. It is unclear if the effects seen by administration of the medication are due to bronchodilation or its anti-inflammatory properties. [155]

6.3 *Additional Treatment Options*

Several other treatment options are available and include alpha-2 adrenergic receptor agonists, anticholinerigics, furosemide, lidocaine, and oxygen therapy. Although these treatments have potential benefits, their use is often limited due to short duration of action, side effects or difficulty in farm administration.

Xylazine, a commonly used equine sedative, is an alpha-2 adrenergic receptor agonist that causes presynaptic inhibition of cholinergic nerves innervating the distal portions of the bronchi of horses. [156] In clinically affected horses, xylazine decreases pulmonary resistance and increases dynamic compliance. [157]

Atropine and ipratropium bromide are anticholinergic drugs that act as an antagonist for the muscarinic acetylcholine receptor. In clinically RAO-affected horses, atropine reduces pulmonary resistance, but does not change dynamic compliance. [13] A decrease in gastrointestinal mobility resulting in colic is a potential side effect of atropine. Ipratropium bromide provides bronchodilation for four hours without side effects when 50 micrograms are administered by inhalation to horses.[49, 139, 158, 159] It does not have the same potential systemic side effects as atropine as it is not absorbed systemically.

Furosemide is a loop diuretic; however its effect on the RAO-affected horses is likely mediated by its effect on prostanoids. [160] Furosemide induces a reduction in pulmonary resistance and increase in dynamic compliance.

Lidocaine is a local anesthetic used to decrease bronchospasm in humans. Although this medication has not been evaluated in horses as a treatment for RAO, it is often included in broncho-alveolar lavages to decrease coughing during the procedure.

Oxygen therapy may be included in acutely affected horses with dyspnea or hypoxemia. Supplemental oxygen improves the partial pressure of oxygen in RAO-affected horses, but the partial pressures remains lower than normal horses. [161]

7. STUDY JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

Recurrent Airway Obstruction, RAO, is a common, incurable disease of horses. Affected horses experience bronchoconstriction, neutrophilic airway inflammation, and mucus accumulation in response to specific airway irritants and allergens. Although the inciting cause of the disease is likely multi-factorial, exposure to hay, including hay molds, is a commonly recognized factor in developing acute exacerbations of RAO and environmental management, including the removal of hay from the diet, is often required for treatment and prevention. Other feeding alternatives, such as complete pelleted diets and wetting hay, have been proposed; however, these have their own limitations.

Steaming hay has been shown to dramatically reduce the number of viable fungal colonies in hay. In addition, the effect of heat on protein would cause protein denaturation. It is not known if the reduction in viable colonies and protein denaturation also leads to a reduction in the antigenic potential and the subsequent clinical response post-exposure. It is possible that mold related antigens could continue to provoke an airway response in sensitized horses.

Our hypotheses were:

1. Steaming hay would reduce the number of viable bacterial and fungal colonies, including two fungal species known to induce clinical signs in RAO-affected horses (*Aspergillus spp.* and *Fusarium spp.*).

2. Steaming hay would reduce clinical parameters and measures of airway inflammation in RAO-affected horses compared to horses fed dry hay.

Our objectives were:

1. Measure clinical parameters, pulmonary function, and determine tracheal mucous volume (as viewed by endoscopic evaluation) of RAO-affected horses fed either dry or steamed hay to determine if the process of steaming attenuates respiratory compromise associated with feeding hay in heavey horses.
2. Measure the cell composition of airway secretions collected by bronchoalveolar lavage from RAO-affected horses fed either dry or steamed hay to determine if the process of steaming attenuates airway inflammation associated with feeding hay to heavey horses.
3. Measure the bacterial and fungal colony growth on steamed and dry hay that is fed to RAO-affected horses to determine in clinical response of horses relates to the density of viable molds and bacteria in the hay.

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CHAPTER 2: COMPARISON OF AIRWAY RESPONSE OF RECURRENT AIRWAY OBSTRUCTION AFFECTED-HORSES FED STEAMED VERSUS NON-STEAMED HAY

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Short Title: RAO-affected Horses' Response to Steamed Hay (max 6 words)

Keywords: horse, heaves, fungus, hay (max 4 words)

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Abbreviations:

RAO	Recurrent Airway Obstruction
PDA	Potato dextrose agar media
ASP	*Aspergillus spp.*-selective media
FUS	*Fusarium spp.*-selective media
BAL	Bronchoalveolar lavage
ΔPpl_{max}	Maximal change in pulmonary pleural pressure during tidal breathing
CFU	Colony Forming Unit
DPBS	Dulbecco's Phosphate Buffered Solution

Animals were housed and data was collected at Virginia-Maryland Regional College of Veterinary Medicine on the campus of Virginia Polytechnic Institute and State University in Blacksburg, VA. The study was supported by a grant provided by HayGain North America, a division of Jiffy Steamer Equine Division. This research was presented as a poster at the 2012 ACVIM Forum in New Orleans, LA. The authors gratefully acknowledge the owners of the horses used in the study, the technical staff of the Virginia-Maryland Regional College of Veterinary Medicine Veterinary Teaching Hospital (VMRCVM VTH) and the student volunteers from Radford University and Virginia Tech.

Abstract

Background – Recurrent Airway Obstruction (RAO)-affected horses experience bronchoconstriction and airway inflammation in response to inhalation of aerosolized irritants including hay molds. Steaming hay reduces viable fungal content, but the effect on the antigenic potential of hay has not been investigated.

Hypothesis/Objectives – The clinical and airway response of RAO-affected horses to hay exposure will be reduced by steaming hay prior to feeding and this reduction will coincide with decreased hay fungal content.

Animals – 6 privately owned RAO-affected horses in clinical remission.

Methods – Two-way cross-over prospective study. Hay was steamed using a commercial hay-steamer. Clinical assessment was performed daily. Full assessment, including upper airway endoscopy, assignment of mucus scores and measurement of maximal change in pleural pressure, was performed on days 1, 5, and 10. Bronchial fluid sampling and cytology were performed on days 1 and 10. Hay core samples were collected pre- and post-steaming and cultured to determine fungal and bacterial concentrations.

Results – Horses fed steamed hay had less increase in abdominal effort ($p= 0.0647$) and total clinical scores ($p=0.0088$), fewer neutrophils per ml of broncho-alveolar fluid ($p=0.1434$), and higher weight gain ($p=0.0649$) compared to horses fed dry hay. Fungal and bacterial colony forming units were significantly reduced by steaming hay.

Conclusions and clinical importance –Steaming hay before feeding serves as an adjunct therapy for reducing RAO-affected horses' response to hay.

Recurrent Airway Obstruction (also known as RAO, Heaves or Chronic Obstructive Pulmonary Disease) is a common, incurable disease that can affect up to 50% of horses in some populations[1]. Affected horses experience bronchoconstriction, neutrophilic airway inflammation, and mucus accumulation in response to exposure of specific airway irritants and allergens. [2] Diagnosis is based on history and clinical signs in conjunction with cytological evaluation of bronchoalveolar lavage fluid revealing a non-septic neutrophilia (>25% neutrophils) in clinically-affected RAO horses. [3]

The inciting cause of airway inflammation is likely multi-factorial and involves antigen stimulating dust, bacteria, endotoxin, noxious gases, and molds.[4, 5] Exposure to hay molds, such as *Aspergillus fumigatus* and *Fusarium spp*, has been shown to induce severe clinical signs in RAO-affected horses [5-11]. *Aspergillus* species have been consistently found in hay and exposing RAO affected horses to this mold has been shown to induce an increase in ΔPpl_{max}. [10] Environmental management, such as moving affected horses to a low dust environment and the removal of hay from the diet, has been shown to be a key factor in treating RAO horses and maintaining them in clinical remission. [12, 13]

Previous studies have demonstrated that steaming hay reduces the viable mold spore content from over 7,775,000 spores per gram of hay pre-steaming to 0 spores post-steaming [14, 15]. However, it is not known whether killing the mold also leads to a reduction in antigenic potential or clinical response post exposure. Wide-spread use of killed viral and bacterial antigens as vaccines has demonstrated the ability of these antigens to retain the potential to stimulate an inflammatory response. Horses have been shown to mount an immune response to tetanus toxoid, inactivated equine influenza and inactivated equine herpesvirus 1 and 4 vaccines. [16-19] Thus, it is possible that while steaming reduces live mold content in hay, the presence of mold related antigens could continue to provoke an airway response in sensitized horses.

The purpose of this study was to evaluate and compare clinical parameters and measures of airway inflammation in RAO-affected horses fed non-steamed hay to the response of horses fed steamed hay to determine if steaming reduced airway irritation and pulmonary compromise. In addition, the effect of steaming on fungal and bacterial concentrations in hay was assessed. In contrast to previous studies,

selective media for two known pathogenic fungi, *Aspergillus spp.* and *Fusarium spp.*, was utilized in order to minimize bacterial overgrowth that might inhibit fungal growth. We hypothesized that the response of RAO-affected horses to hay exposure would be reduced by steaming hay prior to feeding and this reduction would coincide with decreased hay fungal content.

MATERIALS and METHODS

ANIMALS

Six adult horses (mean age 19 years , range 14 – 25 years) of mixed breeds (3 Quarter Horse, 1 Appaloosa, 1 Racking Horse and 1 mixed) and sex (4 mares and 2 geldings) with confirmed Recurrent Airway Obstruction (RAO) were used in the study. All horses were privately owned and previously diagnosed with RAO at the VMRCVM Veterinary Teaching Hospital (VTH). The diagnosis of RAO was based upon history, physical examination, airway endoscopic evaluation, tracheal wash cytology and culture or bronchoalveolar cytology, thoracic ultrasound, and thoracic radiographs. Horses were also known to develop clinical signs of RAO when housed in a barn and fed hay, and to demonstrate resolution of clinical signs when housed on pasture and/or treated with dexamethasone. Only horses that had been on pasture for a minimum of 6 months prior to the start of the study were included. As part of the final selection process, horses underwent a physical examination and were assigned RAO clinical scores as described later in the material and methods section. For inclusion in this study, horses were required to have a normal physical examination and clinical score (≤ 4) at the time of this examination. Written consent for use was obtained from the owners for all animals that participated in the trial.

The study was conducted over the month of February at the VTH to facilitate steaming and quantification of hay, water, and mineral ingestion. Horses were housed in the hospital in 14 x 14 foot stalls with mat flooring and cinderblock walls. Horses were bedded on commercially packaged, low dust pine shavings and turned out for 1 hour daily during the period that feed was weighed back and stalls were cleaned. Prior to the start of each feeding trial, horses were allowed a five day acclimation period, during which time they were fed grain[a] and chopped, pre-packaged hay[b]. They were also dewormed with

moxidectin and their teeth were floated before the start of the study. All treatments and procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee.

Prior to initiation of the study, the horses were randomly divided into two groups of three horses and fed steamed or non-steamed hay using a two-way cross-over study design with a five day rest period between treatment periods. Each horse had free access to water and a mineral supplement and daily intake of each was quantified. Intake of each was recorded daily. In addition, the horses were fed one pound of grain[a] twice daily. Moderately dusty alfalfa hay was steamed using a commercial hay steamer[c] following manufacturer's guidelines. The hay was fed in a manner to allow free choice consumption. The amounts of hay, grain and mineral mix consumed were quantified based on weight, and water consumption was measured based on volume. The volume of water and weight of feed stuffs were recorded at the beginning of every 24 hour period. Water consumption was determined based on the cumulative amount of water required to re-fill graduated buckets to a level of 16 liters over 24 hours, while feed consumption was determined by weighing back the residual grain, hay and mineral mix amounts and subtracting those weights from the amount that was placed in the stall the previous morning or at the beginning of the feeding period. On all days, horses consumed the entire portion of grain they were offered and no additional analysis of this portion of the diet was performed. To facilitate measurement of residual hay, the hay was fed in a large tub which significantly limited hay loss, and any residual stems or leaves were handpicked from the stall prior to cleaning each morning.

Daily evaluation included a physical examination (temperature, heart rate, respiratory rate, mucous membrane color, capillary refill time, and attitude) of each horse and assignment of a clinical score (abdominal effort and nostril flare). On days 1, 5, and 10 of each period, the horses were weighed and assigned a body condition score using the scale of 1 to 9 described by Henneke in 1983.[20] On these days, the horses also received an airway endoscopic evaluation with assignment of a tracheal mucus score and assessment of pulmonary function testing (see details below). On days 1 and 10 of each period,

a bronchoalveolar lavage (BAL) was performed and the recovered fluid was used to assess lower airway inflammation as described in the following sections.

CLINICAL SCORES

Clinical scores were assigned daily utilizing a nostril flare and abdominal press score (NAPS) using as previously described by Robinson, et al., in 2000 and Rush, et al, in 1998.[21, 22] Horses were assigned a score ranging from 1 to 4 for abdominal press and effort and a score ranging from 1 to 4 for nostril flare. The scores were added to determine a total clinical score. (See table 1 for scoring criteria). Scores were assigned by 2 individuals that were blinded to the treatment the horses were currently receiving.

PULMONARY FUNCTION TESTING

Pleural pressure changes were measured on non-sedated horses by means of an esophageal balloon (10 cm long, 3.5 cm perimeter and 0.06 cm wall thickness) sealed over the end of a polypropylene catheter (3 mm internal diameter and 4.4 mm external diameter). The tubing was passed into the distal third of the esophagus and attached to a very low range differential pressure transducer[d] that was calibrated prior to each sampling day by means of a water manometer. The position of the esophageal balloon was adjusted to obtain the maximal change in pleural pressure during tidal breathing. Respiratory flow was measured using a pneumotachograph[e] connected to an airtight face mask and coupled to a differential pressure transducer[f] that provided a signal proportional to flow. The pneumotachograph transducer system was calibrated using a 3 L calibrated syringe. Flow and pleural pressure during breathing were processed by a lung function computer[e]. At each data collection time, values obtained over one minute were averaged.

ENDOSCOPY and TRACHEAL MUCUS SCORES

On days 1, 5 and 10 of each period, the horses were sedated with butorphanol tartrate[g] (0.01 mg/kg) and detomidine hydrochloride[h] (0.01 mg/kg) intravenously. A one-meter video endoscope was passed through the horse's nares to the rostral 1/3 of the trachea. The visible amount of mucus in the trachea was assigned a score of 0 to 5 based on a previously described system (Table 2).[23]

BRONCHOALVEOLAR LAVAGE COLLECTION and CYTOLOGY

Bronchoalveolar lavage was performed on days 1 and 10 of each treatment period. Following intravenous sedation and endoscopic evaluation, a 3-meter bronchoalveolar lavage tube with a cuff[i] was passed through the nares, larynx, and trachea and into the airways until the tube wedged in a distal bronchus. The cuff was inflated and a total of 250 mls of warm saline was infused into the airways. Fluid was aspirated following infusion of 2 aliquots of 100 mls and 1 aliquot of 50 mls. The recovered fluid was pooled and the amount recovered was recorded. The samples were mixed, then equally divided into 50 milliliter conical tubes and centrifuged at 300 x g for 15 minutes. The resulting supernatant was discarded and the cell pellet was washed twice with 30 mls of sterile Dulbecco's Phosphate Buffered Saline (DPBS) (500 x g, 8 minutes). Following removal of the final supernatant, the pellet was resuspended in 500 ul of sterile DPBS. The total nucleated cell concentration per milliliter was determined by an automated cell counter[j] and the total number of nucleated cells was calculated by multiplying the concentration by the volume of fluid in which the cell pellet was resuspended. To calculate the number of cells per ml of recovered BAL fluid, the total cell number was divided by the volume of BAL fluid recovered from the lavage. To determine the distribution of cells in the BAL fluid, a cytospin slide[k] was prepared and stained with a modified Wrights stain[l]. Three hundred cells were counted for each sample by two individuals, one of whom was blinded to the treatment groups, and the differential for each cell population was expressed as a percentage based on the average counts of the two examiners. The total number of each cell population was determined by multiplying the total number of cells by the percentage represented by that population. The number of each cell population (e.g.

neutrophils/ml) was calculated by dividing the total number of cells in that population (e.g. total neutrophils) by the volume of BAL fluid recovered during the lavage.

HAY ANALYSIS

Five core samples were taken from the short end of 6 rectangular hay bales prior to steaming and the opposite short end of the each bale post-steaming (Figure 1). The five core samples from each bale were mixed together to form one sample for analysis.

Three media were used for bacterial and fungal cultures - a non-selective potato-dextrose agar (PDA), an *Aspergillus spp.*-selective media (ASP) and a *Fusarium spp.*-selective media (FUS). The PDA media was composed of 1000 milliliters distilled water, 6 g potato dextrose broth and 15 g BD Bacto Agar[l]. The media was made in 1 liter quantities in a 2 liter Erlenmeyer flask and a stir bar was used on a medium setting during the mixing process. The components were mixed until they formed a solution. The flask was maintained on a warming plate during mixing and the solution was not allowed to boil. The media was autoclaved on a wet (liquid) cycle at 121 degrees Celsius for 40 minutes on slow exhaust to achieve a pressure of 15-20 psi. The media was then poured into 4 inch plates and allowed to cool for 24 hours before being placed in cold storage at 4 degrees Celsius. The ASP media was composed of 1000 mls distilled water, 15 g BD Bacto Agar[m], 20 g yeast extract, 10 g peptone, 0.5 g ferric ammonium citrate, and 1 ml of a 0.2% stock solution of dicloran (0.2 g dicloran with 100 mls 99% ethanol). After the solution was mixed and autoclaved as previously described, 0.1 g chloramphenical dissolved in 10 mls of sterile distilled water was added to the media prior to pouring into plates. FUS media was composed of 1000 mls distilled water, 15 g BD Bacto Agar[m], 15 g peptone, 1 g potassium phosphate, 0.5 g magnesium sulfate, and 1 g Terrachlor[n]. The components were mixed and autoclaved as previously described and 1 g streptomycin sulfate and 0.35 g neomycin sulfate dissolved in 10 mls of sterile distilled water were added prior to pouring into plates.

Specific methods for quantifying fungal and bacterial growth in hay samples for the purpose of this study were adapted from previously published information. [24-26] All cultures were performed in

duplicate using wet and dry methodology. For the dry preparations, two quantities (0.05 g and 0.10 g) of hay were applied directly to the media. For the wet preparations, 7.5 grams of hay were added to 375 ml of distilled water to create a 2% (weight to volume) solution which was considered the 1X concentration. Serial dilutions were performed to yield 1:10X and 1:100X concentrations. One hundred microliters of each concentration were applied to the media. These amounts and concentrations of hay were selected based on a the results of a series of pilot studies aimed at determining the optimal concentration that allowed for colony growth over time without compromising the ability to accurately count the colonies (significant overgrowth). Once the samples were applied to the plates, the plates were cultured at room temperature.

Bacterial and fungal growth was counted as colony forming units (CFUs) daily for a total of 5 days. Bacterial CFUs were determined based on growth on PDA only since *Aspergillus spp.* and *Fusarium spp.* selective media contained antibiotics. Fungal CFUs were counted on all medias. If a plate reached greater than 75 CFUs, the number of colonies were deemed too numerous to count and the plate was discarded. The number of colonies were then determined by examining the plate with the next lower concentration (0.05 g for the dry preparation or 1:10X for the wet preparation) and the number of CFUs were multiplied by 2 or 10 respectively to allow statistical analysis of the number of colonies based on 0.10 grams dry preparation or 100 ul wet preparation (CFU/0.1 grams hay and CFU/100 ul of a 2% solution).

STATISTICAL ANALYSIS

One horse was removed from the study after developing severe clinical signs of RAO (clinical score of 8 with tachypnea and tachycardia) and data obtained from this horse was not included in the analysis. Analyses of clinical scores, body weight, pulmonary function testing and mucus scores were performed by repeated measures ANOVA. Analysis of BAL fluid including milliliters recovered, percentage of infused fluid recovered, total cells, total cells per milliliter as well as the percentage, total cells and cells per milliliter of each cell type was performed using a mixed model repeated measures

ANOVA. The differential cell counts of the two authors were compared using a mixed model ANOVA. Bacterial and fungal colony forming units were compared using a Wilcoxon 2 sample test. All analyses were performed using SAS® software. A p-value ≤ 0.05 was considered significant.

RESULTS

Five of the six horses completed both feeding periods. One horse was removed from the study during the first feeding period after developing severe clinical signs of RAO while being fed dry hay; data from this horse was not included in the analysis. This horse responded to treatment and was returned to the owner without clinical signs of disease.

Physical exam parameters including heart rate, respiratory rate and body temperature did not change significantly over the 10 day feeding period for either group. Mean body weight of the horses tended to increase when fed steamed hay from day 1 to day 10 (p=0.0751) and the amount of weight gained by day 10 in horses fed steamed hay tended to be greater (mean gain = 25 lbs, median gain = 31 lbs, p=0.0649) than in horses fed dry hay (mean gain = 6 lbs, median gain = 2 lbs). When measured on an as fed basis, horses tended to eat more steamed hay than dry hay (p=0.1350). However, steaming increased the moisture in the hay by approximately 15%. When measured on a dry matter basis, horses fed steamed hay consumed approximately 21.5 lbs per day while horses fed dry hay consumed 23.4 lbs per day. Horses fed steamed hay also tended to consume more mineral than horses fed dry hay (p=0.0992). There was no difference in water consumption between the groups. (Table 3)

Horses fed dry hay had a significantly higher abdominal effort score when compared to horses fed steamed hay at day 5 (p=0.0072) and tended to have a higher score on day 10 (p=0.0647). The abdominal effort score tended to increase in horses fed dry hay at both day 5 (p=0.0566) and day 10 (p=0.0814). Horses fed dry hay had significantly higher total clinical scores than horses fed steamed hay on day 5 (p=0.0039) and day 10 (p=0.0088). Total clinical score also increased significantly from

baseline on day 5 (p=0.0073) and day 10 (p=0.0181) in horses fed dry hay. This same increase in total clinical score was not observed in horses fed steamed hay. (Table 4 and Figure 2)

Both treatment groups showed an increase in mucus scores from baseline to day 10, but, only horses fed dry hay had a significant increase (p=0.0339). A significant difference was not observed between treatment groups. A significant difference in maximal change in pleural pressure was not seen between the groups at day 1, 5, or 10. Maximal change in pleural pressure did not increase significantly from baseline to day 5 or day 10; however, the change in horses fed dry hay tended to have a higher value on day 10 compared to baseline (p=0.1008). (Table 4)

The volume (mls) of BAL fluid recovered did not differ between days 1 and 10 for either treatment group and there was no difference between groups. A difference was also not seen between treatment periods. The percentage of BAL fluid recovered tended to increase in the group receiving steamed hay from day 1 to day 10 (p=0.1064). Total cells per milliliter of BAL fluid tended to decrease from day 1 to day 10 when fed dry hay (p=0.0575).

The differential cell counts were performed by two individuals and their average counts were not significantly different. Statistical analysis of each cell type was performed using an average of the individual differential cell counts. Neutrophil percentage increased in both groups (dry hay p=0.0240 and steamed hay p=0.0420) from day 1 to day 10. A greater increase (p=0.0053) was observed during feeding period 1 compared to period 2. There was no difference in neutrophil percentage observed between groups. When comparing total neutrophil counts, there tended to be an increase in neutrophils from day 1 to day 10 in the horses fed dry hay (p=0.0562) and a greater number of neutrophils in horses fed dry hay compared to steamed hay at day 10 (p=0.1103). Neutrophils per milliliter tended to increase from day 1 to day 10 in horses fed dry hay (p=0.0773) and tended to be higher in the dry hay group compared to the steamed hay group at day 10 (p=0.1333). Macrophage percentage decreased significantly in both dry hay (p=0.0069) and steamed hay (p=0.0107) groups between day 1 and day 10. A significant difference was not observed between groups. Total macrophages (p=0.0880) and macrophages per milliliter (p=0.0891) tended to decrease from day 1 to day 10 in horses fed dry hay. No significant difference was seen in

lymphocytes between days or groups, but the number of lymphocytes tended to decrease in period 1 and increase in period 2. No significant differences were seen in mast cells or eosinophils. (Table 5)

Significant differences in bacterial colony forming units were seen in the non-selective PDA media. On the wet preparation, significantly less bacterial growth was measured in the steamed hay compared to dry hay and was first observed on day 2 ($p=0.0143$). This difference persisted through days 3 ($p=0.0166$), 4 ($p=0.0166$), and 5 ($p=0.0165$). Results were similar in the dry preparation with steamed hay having less bacterial growth. This was observed as a trend on day 1 ($p=0.0745$). A significant difference was seen on days 2 ($p=0.0404$), 3 ($p=0.0402$), 4 ($p=0.0462$), and 5 ($p=0.0535$). Bacterial colony forming units were only counted on PDA media since the selective media contained antibiotics to retard bacterial growth. (Figure 3 and Table 7)

Analysis of fungal colony forming units using a wet preparation method revealed significantly less growth in the steamed hay on PDA on days 3 ($p=0.0252$), 4 ($p=0.0143$), and 5 ($p=0.0143$) and on the ASP media on days 3 ($p=0.0357$), 4 ($p=0.0154$), and 5 ($p=0.0156$). No difference in growth in the steamed and dry hay was observed in the wet preparation on FUS media due to very little growth. Results were similar in the dry preparation method on PDA with significantly less growth in the steamed hay on days 2 ($p=0.0242$), 3 ($p=0.0165$), 4 ($p=0.0166$), and 5 ($p=0.0171$). There was less growth in steamed hay on the ASP media on days 2 ($p=0.051$) 3 ($p=0.0165$), 4 ($p=0.0171$), and 5 ($p=0.0171$). On FUS media, there was a low number of colonies; however, steamed hay tended to have less growth on days 3 ($p=0.1007$) and 4 ($p=0.0509$) and significantly less growth on day 5 ($p=0.0121$). Results of fungal growth on day 5 with dry preparation are depicted in Table 6. Figure 4 is an example of the fungal growth observed on *Aspergillus spp.*-selective plates.

DISCUSSION

The results of this study indicate that steaming hay reduces the clinical response of RAO-affected horses to being fed hay. When fed steamed hay, horses displayed significantly less abdominal effort and had a lower total clinical score than when fed dry hay. Both groups demonstrated a tendency towards an

increase in airway inflammation as indicated by an increase in tracheal mucus score and the percentage of neutrophils in their BAL fluid on Day 10 of the feeding trial as compared to Day 1 (tables 4 and 5). However, only horses fed dry hay tended to increase the total number of neutrophils ($p=0.056$) and neutrophils per ml of retrieved BAL fluid ($p=0.0773$) between Day 1 and Day 10 of feeding. This difference was not influenced by the amount of fluid retrieved since the volume did not differ significantly between groups or over time. These findings suggest that horses fed dry hay experienced a greater airway inflammatory response over time as compared to horses fed steamed hay. Maximal change in pleural pressure during tidal breathing is a measurement of the work of breathing, and is increased in horses with clinical RAO in response to airway obstruction and diminished lung compliance.[8] Neither treatment group in this study showed a significant increase in ΔPpl_{max} over the 10 day feeding period. However, horses fed dry hay tended to have a higher ΔPpl_{max} on Day 10 compared to baseline (table 4). In total, these findings suggest that steaming hay reduces RAO affected horses response to hay exposure.

Several aspects of this study may have attenuated the potential beneficial effect of steaming. The study was conducted on a small number of horses. The initial design included 6 horses, but the elimination of one horse due to development of severe clinical signs of RAO reduced the final number to five. In almost all cases where differences between the groups approached but did not reach significance, variation between horse responses was large. The impact of this variation on the total data set reduced the possibility of detecting differences using $p < 0.05$.

Horses were selected for inclusion in this study based on initial clinical parameters and scores, and cytological evaluation of BAL fluid was only performed on the first day of the feeding trial. Based on assessment of Day1 samples, both groups had a BAL neutrophilia (greater than 25%). [3] There was no difference in the total number of neutrophils, neutrophils/ml of retrieved BAL fluid or percentage of neutrophils in BAL fluid obtained on Day 1 between feeding groups indicating that significant differences in initial inflammation did not contribute to the tendency for horses fed dry hay to develop a greater neutrophil response after 10 days (Table 5). Previous studies have demonstrated a poor correlation between the number of neutrophils per microliter of BAL fluid and severity of airway

obstruction.[12] The predominant goal of this study was to assess clinical response of RAO-affected horses to steamed hay, and is the justification for using selection criteria that were solely based on the animals' physical examination parameters and clinical scores. Future studies in which assessment of airway inflammation is a more central goal should include BAL fluid assessment as criteria for animal inclusion.

A second aspect of the study design that may have influenced the outcome was the brevity of the rest period between treatment periods. In order to minimize the impact of variation in climate and environment, the rest period between feeding trials was 5 days. During that time, horses were housed on pasture and supplemented with grain and chopped forage. Previous studies have shown that clinical signs and pulmonary function of RAO horses can return to normal within one week or less when affected horses are placed on pasture and removed from hay.[8, 27] In addition, clinical signs of RAO can improve when hay is removed even when the horse remains indoors.[28, 29] Horses were not treated with any medication during the study, and for this reason, a prolonged wash-out period was also not deemed necessary. Statistical evaluation of an effect of treatment period also failed to identify outcomes that were influenced by treatment period with the exception of the total number of cells in the BAL fluid. This value tended to be lower in both groups of horses at the start of the second feeding period as compared to the first. The absence of an effect of treatment period suggests that the brief rest period used in this study did not significantly impact the outcome of the study. However, the effect of steaming may have been more evident if horses were in complete remission (clinical and airway inflammation) at the start of both treatment periods and based on the results of this study, horses would have required a more prolonged rest period between treatments to return to complete remission.

The period of time that horses were exposed to steamed and dry hay was relatively short, yet even with only a ten day exposure, horses fed dry hay experienced a trend towards an increase in clinical score, BAL nucleated cell count, total neutrophil count, and ΔPpl_{max} that was not apparent when horses were fed steamed hay. It is possible that over time, the clinical difference between these two groups may have become increasingly divergent. The response of horses fed steamed versus dry hay for a more prolonged

period should be investigated, as an increased benefit over time would better justify the financial investment associated with purchase of a commercial steamer.

Since the goal of this study was to examine the effect of steaming hay, no other aspect of the horse's environment was modified. Horses were housed in stalls that were made of cement blocks on three sides with wall heights of 14 feet. The fronts of the stalls were also solid block to a height of 4.5 feet, and a mesh wire that extended from a height of 4.5 feet to 14 feet. While horses were visible through this mesh, the stall structure allowed for little ventilation. The floors consisted of cement covered with pour-in flooring, rubber mats and low dust commercial shavings were used to bed the stalls. Horses were moved outside of the stalls when the stalls were cleaned. This management likely minimized horse exposure to aerosolized debris as compared to the debris experienced by a horse bedded on straw, fed hay, and housed in a wooden barn, although measurements of ventilation and debris exposure were not assessed in this study.[30] Modifying the environment alone has been shown to dramatically decrease the clinical signs of RAO in affected horses and is the mainstay of treatment.[12, 13, 31] Coupling hay steaming with other environmental modifications may be a more effective (and practical) application of this technology.

The reduction in clinical disease associated with the steamed hay diet coincided with a reduction in mold and bacterial growth in samples of hay placed in culture as compared to samples from the same hay bale that were collected and placed in culture prior to steaming. The method employed in this study has not been previously reported and was not intended to provide an absolute evaluation of the bacterial or fungal concentrations in hay. Instead, it was applied as a comparative method to determine if fungal and bacterial concentrations were reduced by steaming. While there are many methods for detecting evidence of fungi in hay, feces, gut contents, and soil, the goal of this study was to compare fungal growth in hay before and after steaming for the purpose of confirming that previously reported reduction in mold growth was repeated in our study.[25, 26, 32, 33] An additional goal was to demonstrate that a reduction in viable fungi, like *Aspergillus spp.*, coincided with an attenuation of response in RAO affected horses. Hay molds, including *Aspergillus spp.* and *Fusarium spp.*, have been implicated as a cause of the

clinical signs of RAO.[6-8] The tertiary (or 3-dimensional) structure of proteins can become denatured starting at 122 °F.[34] When steaming hay following the manufacturer's guidelines, the hay reached a temperature of 160 °F. Based on this information, it seems likely that steaming hay could denature the proteins of the fungal organisms and the change in structure could cause a decrease in the antigenic potential.

Steaming may also result in an overall reduction in the dose of inhaled bacteria, fungus, and/or other irritating aerosolized debris. Steaming increases the weight of an average rectangular bale by 15 to 20%. This increased weight is due to an increase in water content in the hay. The aerodynamic potential of dust can be reduced by wetting, and a reduction in the exposure dose of any or all of the antigenic or irritating particles in hay may have been the reason for the attenuated response observed in RAO horses fed steamed hay as compared to dry. No attempt was made in this study to measure or directly classify inhaled particulate matter to which horses were exposed.

While airway inflammation is most commonly associated with exposure to respirable debris, studies of human asthmatics have shown a relationship between gut flora, diet, and airway inflammation.[35-37] Activated inflammatory cells and inflammatory signaling events that are initiated in the gut mucosa can produce similar effects in the airway lining. Steaming results in a decrease in the viable bacteria and fungi (such as *Aspergillus spp.*) in hay and this reduction may have a secondary impact on the gut flora and/or antigenic profile of hay after ingestion.

Horses in this study experienced a significantly greater increase in body weight when fed free choice steamed hay as compared to dry hay. The increased water content of steamed hay may have increased the water content of gastrointestinal contents resulting in the increase in body weight. However, the pattern of weight gain was gradual throughout the 10 day feeding period. In order for the weight gain to be attributed solely to increased water intake, we would have expected the most significant weight gain would to earlier in the feeding period followed by stabilization of body weight. In addition, horses fed steamed hay ate approximately 13% more hay as fed than when fed dry hay. Steaming

increased hay moisture content from a mean of 9.9% (dry hay) to a mean of 26% (steamed hay) and bale weight increased by approximately 25%. When adjusting for these changes, horse fed steamed hay actually consumed less on a dry matter basis (21.5 lbs) as compared to horses eating dry hay (23.4 lbs). It is possible that steaming improved the digestibility of the hay resulting in an increase in body weight despite a decrease in dry matter intake; however, a digestibility study evaluating of a greater number of horses and examination of weight gain over a longer feeding period is needed to confirm this.

Despite ingesting a greater amount of water in the steamed hay, there was no difference between groups in the amount of water the horses freely drank. The amount of mineral the horses consumed did not differ; however, the mean intake of mineral was higher in horses fed steamed hay as compared to dry hay (41g/day vs. 21 g/day). It is possible that the increased water intake resulted in the horses consuming greater quantities of mineral to maintain adequate homeostasis.

In summary, steaming hay significantly reduced the number of fungal and bacterial CFUs in hay. In addition, horses fed steamed hay showed fewer clinical signs of RAO including significantly lower abdominal effort scores and total clinical scores than when the same horses were fed dry hay. Horses fed steamed hay also tended to have less airway inflammation than horses fed dry hay and better pulmonary function, although further studies are required to confirm this conclusion. These differences were observed in less than ideal circumstances, since no other aspect of the horse's environment was modified and the stalls in which they were housed were not well ventilated. It is likely that greater benefits would be observed when horses with RAO are fed steamed hay as an adjunct treatment to other environmental modifications. In addition, feeding steamed hay may increase the amount (in lbs) of hay ingested on an as fed basis, while reducing the actual dry matter intake required to maintain and/or gain body mass in horses affected with RAO and possibly geriatric horses in general. However, these observations were based on the result of a small number of horses fed steamed or dry hay for a brief period of time (10 days) and further investigation is warranted to confirm the association with feeding steamed hay and weight gain.

[a] Legend'sShow & Pleasure Textured grain, Southern States Cooperative, Richmond, VA
[b] Triple Crown Premium Chopped Blended Alfalfa Forage, Triple Crown Nutrition, Wayzata, MN
[c] HAYGAIN HG-1000, HAYGAIN North America, Jiffy Steamer Equine Division, Union City, TN
[d] Validyne Model DP/45-28, Validyne Engineering Corp, Northridge, CA
[e] Buxco Electronics Inc, Wilmington, NC
[f] Validyne Model DP/45-14, Validyne Engineering Corp, Northridge, CA
[g] Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA
[h] Dormosedan®, Pfizer Animal Health, New York, NY
[i] Bivona, division of Smiths Medical ASD, Inc, Weston, MA
[j] Cellometer Auto T4, Nexcelom Bioscience, LLC, Lawrence, MA
[k] Shandon Cytospin 3, Thermo Electron Corporation, Waltham, MA
[l] Hema-tek 2000, Bayer HealthCare, LLC, Mishawaka, IN
[m] Becton, Dickinson, and Company, Franklin Lakes, NJ
[n] Chemtura USA Corporation, Middlebury, CT

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Table 1: NAPS Scoring System [21, 22]

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

Table 2: Mucous Scoring System[23]

Mucus Grade	Description
0	No visible mucus
1	Singular small blobs
2	Multiple blobs only partially confluent
3	Mucus ventrally confluent
4	Large ventral pool
5	Profuse amounts of mucus occupying more than 25% of the tracheal lumen

Figure 1: Location of hay core samples taken from the short end of a bale.

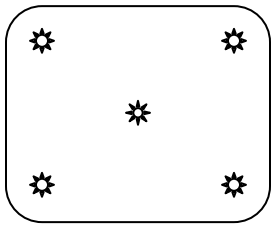


Table 3: Daily Mean Temperature (Fahrenheit), Median Heart Rate, Mean Respiratory Rate, and Mean Body Weight (kilograms), Mean Hay Intake, Mean Water Intake and Median Mineral Intake.

Parameter	Group	DAY									
		1	2	3	4	**5**	6	7	8	9	**10**
T	Dry	**99.3 ± 0.1**	99.4 ± 0.1	99.4 ± 0.1	99.4 ± 0.1	**99.3 ± 0.1**	99.4 ± 0.1	99.2 ± 0.1	99.4 ± 0.0	98.9 ± 0.2	**99.1 ± 0.2**
	Steam	**99.5 ± 0.2**	99.3 ± 0.2	99.3 ± 0.2	99.0 ± 0.1	**99.3 ± 0.1**	99.4 ± 0.1	99.3 ± 0.1	99.4 ± 0.1	99.0 ± 0.2	**99.2 ± 0.1**
HR	Dry	**40 ± 3.9**	40 ± 1.8	36 ± 1.8	40 ± 2.8	**36 ± 2.2**	36 ± 2.2	36 ± 1.8	36 ± 1.8	40 ± 2.2	**36 ± 1.8**
	Steam	**40 ± 2.2**	36 ± 3.3	36 ± 4.4	36 ± 1.8	**36 ± 3.3**	36 ± 4.6	36 ± 4.6	36 ± 3.6	36 ± 4.9	**32 ± 6.1**
RR	Dry	**32 ± 7.3**	28 ± 5.1	28 ± 2.2	29 ± 2.3	**26 ± 1.6**	26 ± 1.6	26 ± 2.0	26 ± 1.6	27 ± 1.5	**29 ± 2.9**
	Steam	**34 ± 6.3**	27 ± 3.4	30 ± 2.7	29 ± 1.5	**29 ± 2.7**	28 ± 2.5	31 ± 3.9	31 ± 3.9	26 ± 1.6	**28 ± 2.2**
BW	Dry	**438 ± 14.8**	--	--	--	**439 ± 16.2**	--	--	--	--	**440 ± 13.5**
	Steam	**434 ± 14.3**	--	--	--	**438 ± 13.2**	--	--	--	--	**446 ± 16.4**
Hay	Dry	**25 ± 2.9**	26 ± 3.5	25 ± 3.8	26 ± 2.9	**26 ± 2.2[1]**	29 ± 2.1	24 ± 3.0	25 ± 3.1	28 ± 2.5	--
	Steam	**25 ± 3.2**	28 ± 3.8	27 ± 5.0	29 ± 3.5	**30 ± 3.9[a1]**	32 ± 3.0	30 ± 3.5	31 ± 3.5	31 ± 2.5[a]	--
Water	Dry	**23 ± 2.6**	29 ± 2.1	39 ± 10.7	38 ± 8.5	**41 ± 10.4**	34 ± 12.8	39 ± 6.6	38 ± 7.1	34 ± 5.3[a]	--
	Steam	**30 ± 7.3**	37 ± 7.0	32 ± 7.3	36 ± 7.9	**38 ± 8.2**	38 ± 7.9	37 ± 6.8	33 ± 6.3	41 ± 9.5[a]	--
Mineral	Dry	**0.0 ± 1.8**	0.5 ± 1.5	0.0 ± 1.3	0.0 ± 1.5	**0.0 ± 0.9**	0.0 ± 1.0	0.0 ± 0.0	0.0 ± 1.8	0.0 ± 0.7	--
	Steam	**0.5 ± 1.7**	3.0 ± 1.6	3.0 ± 1.9	1.5 ± 1.1	**0.0 ± 1.0**	2.0 ± 1.2	0.5 ± 1.6	0.0 ± 0.9	1.5 ± 1.0	--

Day: treatment day of feeding period, Group: type of hay horse received, T: mean body temperature in Fahrenheit ± standard error of the mean, HR: median heart rate in beats per minute ± standard deviation, RR: mean respiratory rate in breaths per minute ± standard error of the mean, BW: mean body weight in kilograms ± standard error of the mean, Hay: mean hay intake in pounds ± standard error of the mean, Water: mean water intake in liters ± standard error of the mean, Mineral: median mineral intake in ounces ± standard deviation.

Complete evaluations were performed on days 1, 5, and 10. T, HR, RR, and BW measured at beginning of each day. Hay, water and mineral measurements are for the following 24 hours. After evaluation on the morning of day 10, the horses began their rest period and hay, water and mineral intake was not included. Statistical analysis was performed comparing days 1, 5, and 10 only. Superscript letter indicates significant change from baseline and superscript number indicates significant difference between groups.

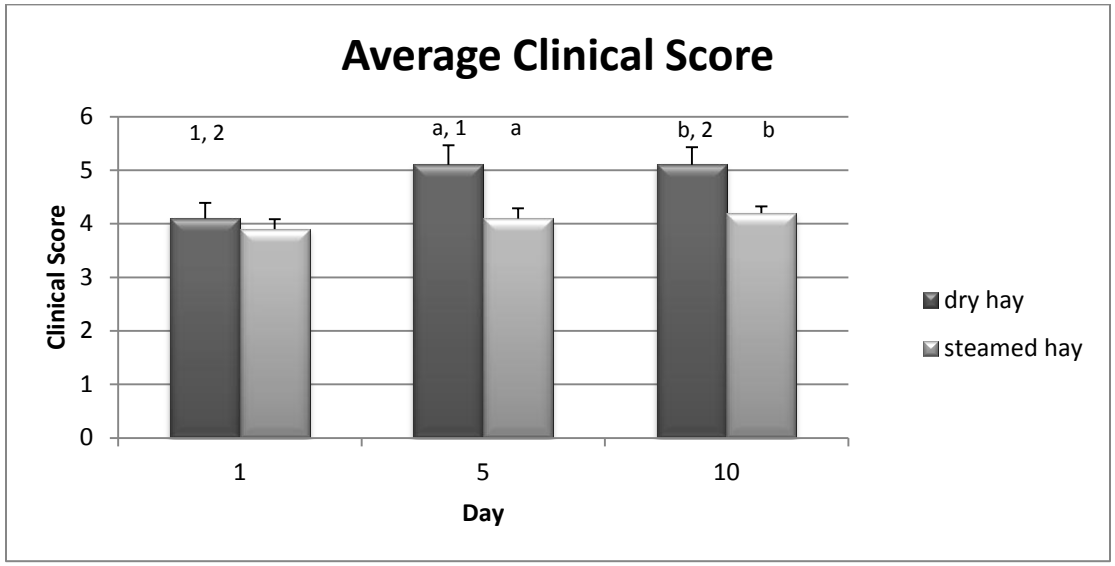
Table 4: Mean values for abdominal effort score, nostril flare score, total clinical score, tracheal mucous score, and maximal change in pulmonary pleural pressure during tidal breathing (on days 1, 5, and 10)

Day	Group	AES	NFS	TCS	TMS	ΔPpl_{max}
1	Dry	2.1 ± 0.24	2.0 ± 0.35	4.1 ± 0.29	1.2 ± 0.49	10.4 ± 3.1
1	Steam	1.9 ± 0.19	2.0 ± 0.00	3.9 ± 0.19	0.9 ± 0.19	9.9 ± 1.2
5	Dry	2.7 ± 0.25[a]	2.5 ± 0.42	5.1 ± 0.37[2c]	1.3 ± 0.25	12.5 ± 3.2
5	Steam	2.0 ± 0.22[a]	2.0 ± 0.22	4.1 ± 0.19[c]	1.2 ± 0.31	12.4 ± 2.1
10	Dry	2.6 ± 0.19	2.5 ± 0.42	5.1 ± 0.33[2d]	2.9 ± 0.46[3]	15.9 ± 2.6
10	Steam	2.2 ± 0.12	2.0 ± 0.22	4.2 ± 0.12[d]	2.4 ± 0.67	13.3 ± 2.5

Day: treatment day of feeding period, Group: type of hay horse received, AES: mean abdominal effort score ± SEM, NFS: median nostril flare score ± SD, TCS: mean total clinical score ± SEM, TMS: mean tracheal mucous score ± SEM, ΔPpl_{max}: mean maximal change in pulmonary pleural pressure during tidal breathing ± SEM.

Same letter superscript indicates significant difference between steamed and dry group, number indicates significant difference from baseline (day 1)

Figure 2: Average total clinical scores on days 1, 5, and 10 for horses on dry and steamed hay



Same numbers and letters indicate significant differences

Table 5: Mean ± SEM of BAL fluid retrieved, percentage recovered, total cells and individual cell type differentials.

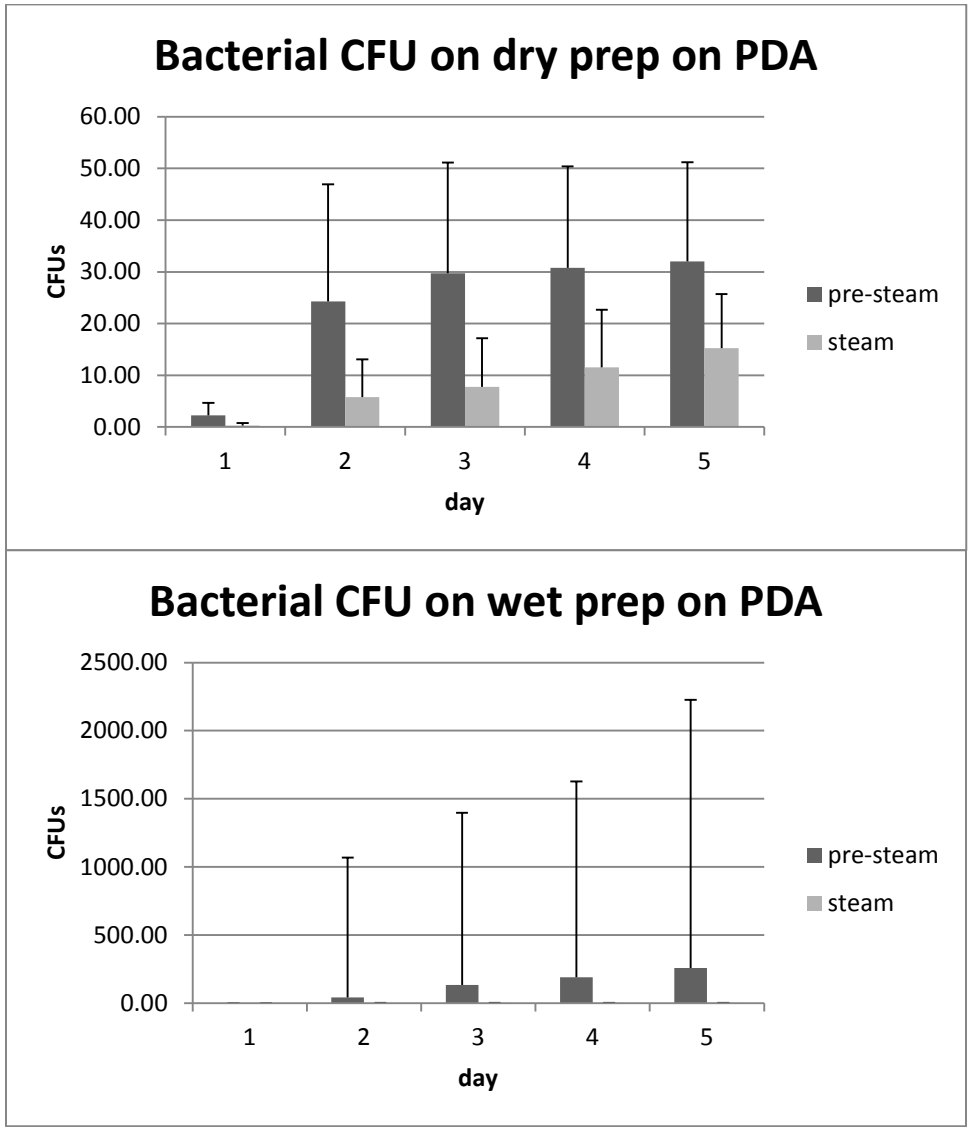
					Neutrophil			Macrophage		
Group	Day	BAL fluid (ml)	% Recovered	Total Cells (x 10^6)	Total (x 10^6)	X 10^3/ml	%	Total (x 10^6)	X 10^3/ml	%
Dry	1	96 ± 12	45.8 ± 4.3	42.2 ± 31.1	4.80 ± 2.5	47.67 ± 23.12	28.4 ± 13.5[2]	9.93 ± 6.90	88.09 ± 61.08	23.6 ± 5.4[4]
Steam	1	107 ± 10	41.3 ± 4.8	27.8 ± 9.6	5.66 ± 1.9	55.53 ± 17.79	28.5 ± 12.3[1]	7.78 ± 3.88	65.15 ± 29.44	25.4 ± 6.6[3]
Dry	10	111 ± 11	43.7 ± 5.0	38.0 ± 15.5	26.7 ± 13.0	275.16 ± 148.23	51.7 ± 12.2[2]	2.31 ± 0.83	22.54 ± 9.56	11.7 ± 3.1[4]
Steam	10	103 ± 8	38.7 ± 4.8	14.7 ± 4.5	7.78 ± 3.6	73.14 ± 33.94	53.1 ± 15.4[1]	1.92 ± 0.79	17.26 ± 6.06	9.3 ± 2.6[3]

		Mast Cell			Lymphocyte			Eosinophil		
Group	Day	Total (x10^6)	x 10^3/ml	%	Total (x10^6)	x 10^3/ml	%	Total (x10^6)	x 10^3/ml	%
Dry	1	0.36 ± 0.25	3.08 ± 1.91	2.5 ± 1.2	25.09 ± 22.35	242.05 ± 199.10	45.3 ± 8.7	0.015 ± 0.009	0.146 ± 0.091	0.17 ± 0.13
Steam	1	1.22 ± 1.00	9.79 ± 7.59	3.5 ± 1.7	13.12 ± 4.95	112.63 ± 39.51	42.5 ± 6.7	0.005 ± 0.005	0.044 ± 0.044	0.10 ± 0.10
Dry	10	0.18 ± 0.07	1.76 ± 0.82	3.0 ± 2.2	8.81 ± 2.70	83.19 ± 28.15	35.7 ± 8.6	0.014 ± 0.013	0.127 ± 0.118	0.20 ± 0.16
Steam	10	0.22 ± 0.09	1.98 ± 0.10	1.5 ± 0.6	4.77 ± 1.71	47.53 ± 18.35	33.6 ± 14.5	0.019 ± 0.010	0.201 ± 0.114	0.17 ± 0.09

Group: type of hay horses were being fed, Day: number of day in study

Same superscript number indicates significant change from day 1 to day 10 within the treatment group

Figure 3: Fungal and bacterial growth on non-selective potato dextrose agar media. Data shown as median ± SD.



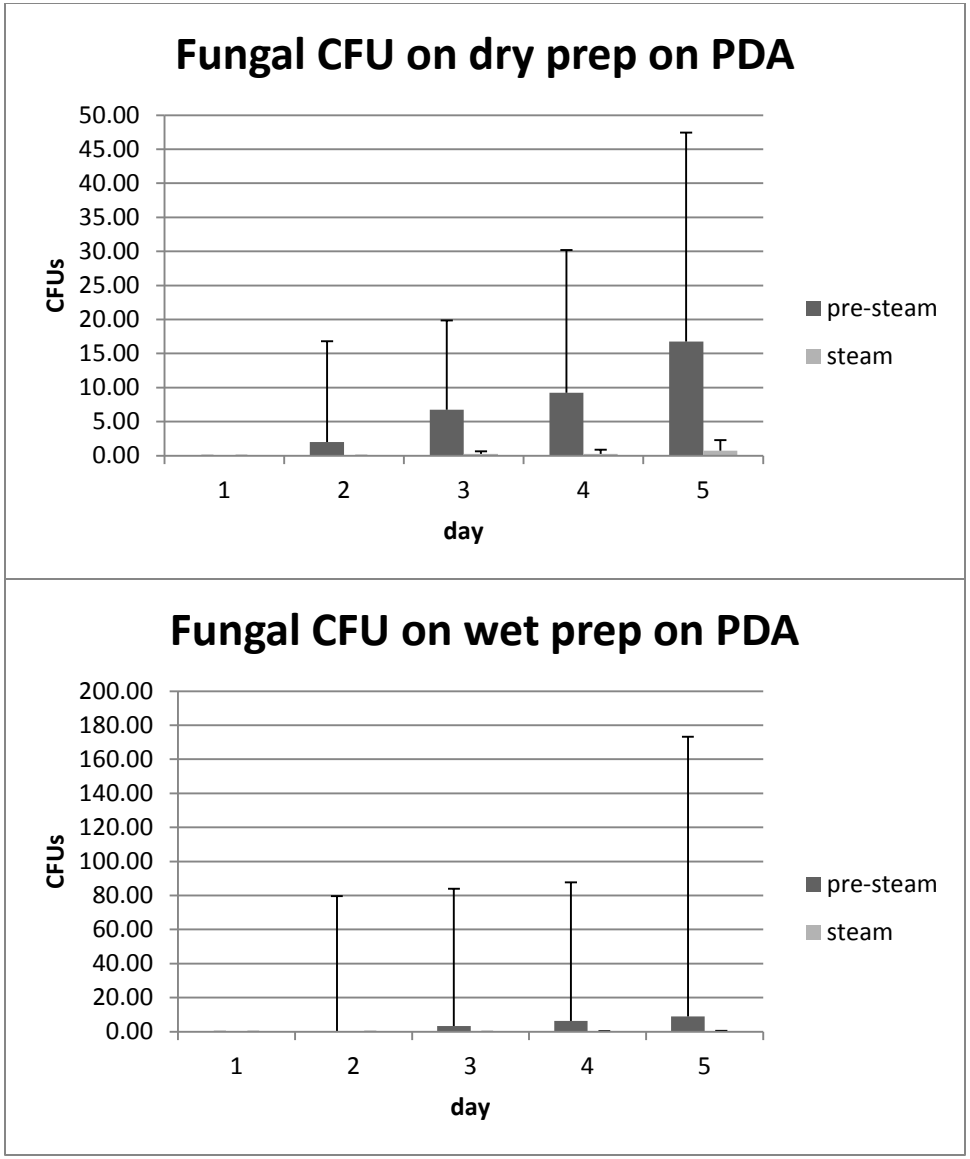

Fungal CFU on dry prep on PDA
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
CFUs
1
2
3
4
5
day
pre-steam
steam
Fungal CFU on wet prep on PDA
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CFUs
1
2
3
4
5
day
pre-steam
steam

Table 6: Median fungal growth in colony forming units on day 5 on non-selective potato dextrose agar media (PDA), *Aspergillus*-selective media (ASP), and *Fusarium*-selective media (FUS).

Hay	Media	Prep	Median	Minimum	Maximum	1st quartile	3rd quartile	IQR
Pre-steamed	PDA	dry	16.8[a]	9.0	90.0	12.5	32.0	19.5
Steamed	PDA	dry	0.8[a]	0.0	4.0	0.0	2.0	2.0
Pre-steamed	PDA	wet	9.0[b]	3.0	400.0	5.5	200.0	194.5
Steamed	PDA	wet	0.0[b]	0.0	1.0	0.0	0.0	0.0
Pre-steamed	ASP	dry	37.0[c]	17.0	150.0	22.0	107.0	85.0
Steamed	ASP	dry	3.0[c]	0.0	10.5	0.5	7.5	7.0
Pre-steamed	ASP	wet	11.3[d]	2.5	410.0	4.5	50.0	45.5
Steamed	ASP	wet	0.0[d]	0.0	0.5	0.0	0.5	0.5
Pre-steamed	FUS	dry	5.8[e]	0.5	18.0	0.5	12.5	12.0
Steamed	FUS	dry	0.0[e]	0.0	0.0	0.0	0.0	0.0
Pre-steamed	FUS	wet	0.0	0.0	0.0	0.0	0.0	0.0
Steamed	FUS	wet	0.0	0.0	0.0	0.0	0.0	0.0

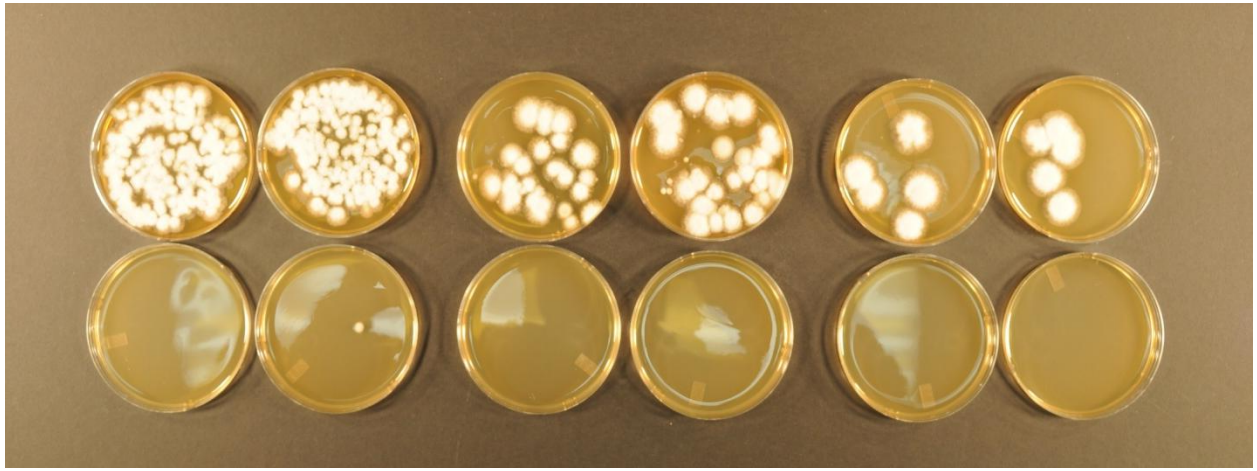
Same letter superscript indicates significant difference between pre-steamed and steamed hay. IQR is the interquartile range.

Table 7: Median bacterial growth on day 5 in colony forming units on non-selective potato dextrose agar media (PDA).

Hay	Media	Prep	Median	Minimum	Maximum	1st quartile	3rd quartile	IQR
Pre-steamed	PDA	dry	32.0	26.0	76.5	28.0	46.5	18.5
Steamed	PDA	dry	15.3	4.0	31.0	9.5	27.5	18.0
Pre-steamed	PDA	wet	257.5[a]	7.5	5000.0	55.0	380.0	325.0
Steamed	PDA	wet	0.5[a]	0.0	4.0	0.5	4.0	3.5

Same letter superscript indicates significant difference between pre-steamed and steamed hay. IQR is the interquartile range.

Figure 4: Growth on *Aspergillus*-selective media using wet preparation methods on day 5 of culture growth. The top row is pre-steamed hay and the bottom row is steamed hay. Concentrations, decreasing from left to right, are 1X, 1:10X and 1:100X. Duplicates of each dilution are shown.



CHAPTER 3: MATERIALS AND METHODS

3.1 *Pulmonary Function Testing*

- Pleural pressure changes are measured on non-sedated horses.
- An esophageal balloon (10 cm long, 3.5 cm perimeter and 0.06 cm wall thickness) sealed over the end of a polypropylene catheter (3 mm internal diameter and 4.4 mm external diameter) is passed into the distal third of the esophagus and attached to a very low range differential pressure transducer (Validyne Model DP/45-28, Validyne Engineering Corp, Northridge, CA) that is calibrated prior to each sampling day by means of a water manometer.
- The position of the esophageal balloon is adjusted to obtain the maximal change in pleural pressure during tidal breathing.
- Respiratory flow is measured using a pneumotachograph (Buxco Electronics Inc, Wilmington, NC) connected to an airtight face mask and coupled to a differential pressure transducer (Validyne Model DP/45-14, Validyne Engineering Corp, Northridge, CA) that provides a signal proportional to flow.
- The pneumotachograph transducer system is calibrated using a 3 L calibrated syringe.
- Flow and pleural pressure during breathing are processed by a lung function computer (Buxco Electronics Inc, Wilmington, NC).
- At each data collection time, values obtained over one minute are averaged.

3.2 *Endoscopy and Tracheal Mucous Scores*

- Horses are sedated with butorphanol tartrate (0.01 mg/kg) and detomidine hydrochloride (0.01 mg/kg) intravenously.
- A one meter scope is passed through the horse's nares and ventral nasal meatus, through the pharynx and larynx to the rostral 1/3 of the trachea.
- The visible amount of mucus in the trachea is assigned a score of 0 to 5 based on a previously described system.[1] The scores are defined in the following table.

Table 1: Tracheal Mucous Scoring System

Mucus Grade	Description
0	No visible mucus
1	Singular small blobs
2	Multiple blobs only partially confluent
3	Mucus ventrally confluent
4	Large ventral pool
5	Profuse amounts of mucus occupying more than 25% of the tracheal lumen

3.3 *Bronchoalveolar Lavage Fluid Collection and Cytology*

- Sedate the horse with butorphanol tartrate (0.01 mg/kg) and detomidine hydrochloride (0.01 mg/kg) administered intravenously.
- A 3 meter bronchoalveolar lavage tube with a cuff (Bivona, division of Smiths Medical ASD, Inc, Weston, MA) is passed through the nares, ventral meatus of the nasal passage, larynx, and trachea into the airways until the tube is wedged in the distal bronchus.
- The cuff is inflated with sufficient quantity of air to form a seal.
- A total of 250 mls of warm saline is infused into the airways. Fluid is aspirated following 2 aliquots of 100 mls and 1 aliquot of 50 mls.
- The recovered fluid is pooled and the amount recovered is recorded.
- The samples are mixed and then equally alliquotted into 50 milliliter conical tubes.
- The conical tubes are centrifuged at 300 x g for 15 minutes.
- The resulting supernatant is discarded and the cell pellet is washed twice in 30 mls of sterile DPBS (500 x g, 8 minutes).
- Following removal of the final supernatant, the pellet is resuspended in 500 ul of sterile DPBS.
- The total nucleated cell count per milliliter is determined by an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience, LLC, Lawrence, MA) and the total number of cells is calculated by multiplying the cell number per ml times the volume of fluid in which the cell pellet is resuspended. To calculate the number of cells per ml of recovered BAL fluid, the total cell number is divided by the volume of BAL fluid recovered from the lavage.

- To determine the distribution of cells in the BAL fluid, a cytospin slide (Shandon Cytospin 3, Thermo Electron Corporation, Waltham, MA) is prepared following manufacturer's guidelines.
- The slide is stained with a modified Wrights stain using a commercial stainer (Hema-tek 2000, Bayer HealthCare, LLC, Mishawaka, IN).
- A total of three hundred cells (3 x 100) are counted for each sample by two individuals.
- The differential for each cell population is expressed as a percentage based on the average counts of the two examiners. The total number of each cell population is determined by multiplying the total number of cells by the percentage represented by that population. The number of each cell population (neutrophils/ml for example) is calculated by dividing the total number of cells in that population (total neutrophils for example) by the volume of BAL fluid recovered during the lavage.

3.4 *Media Preparation for Fungal and Bacterial Cultures*

- Three media are used for bacterial and fungal cultures - a non-selective potato-dextrose agar (PDA), an *Aspergillus spp.*-selective media (ASP) and a *Fusarium spp.*-selective media (FUS).
- The potato-dextrose agar is composed of 1000 milliliters distilled water, 6 g potato dextrose broth and 15 g BD Bacto Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ).
    - The media is made in 1 liter quantities in a 2 liter Erlenmeyer flask and a stir bar is used on a medium setting during the mixing process. The flask is maintained on a warming plate during mixing and the solution was not allowed to boil.
    - The components are mixed until they form a solution.
    - The media is autoclaved on a wet (liquid) cycle at 121 degrees Celsius for 40 minutes on slow exhaust to achieve a pressure of 15-20 psi.
    - The media is then poured into 4 inch plates and allowed to cool for 24 hours.
    - After 24 hours, the plates are placed in cold storage at 4 degrees Celsius.

- The *Aspergillus spp.*-selective media is composed of 1000 mls distilled water, 15 g BD Bacto Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ), 20 g yeast extract, 10 g peptone, 0.5 g ferric ammonium citrate, and 1 ml of a 0.2% stock solution of dicloran (0.2 g dicloran with 100 mls 99% ethanol). In addition, 0.1 g of chloramphenical will be needed after autoclaving the original mixture.
    - After the solution is mixed and autoclaved as previously described in the potato-dextrose agar, 0.1 g chloramphenical is dissolved in 10 mls of sterile distilled water and then added to the media prior to pouring into plates.
    - The solution is poured into 4 inch plates and allowed to cool for 24 hours.
    - After 24 hours, the plates are placed in cold storage at 4 degrees Celsius.
- *Fusarium spp,*-selective media is composed of 1000 mls distilled water, 15 g BD Bacto Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ), 15 g peptone, 1 g potassium phosphate, 0.5 g magnesium sulfate, and 1 g Terrachlor (Chemtura USA Corporation, Middlebury, CT). In addition, 1 g of streptomycin sulfate and 0.35 g of neomycin sulfate will be needed after autoclaving the original mixture.
    - After the solution is mixed and autoclaved as previously described in the potato-dextrose agar, 1 g streptomycin sulfate and 0.35 g neomycin sulfate are dissolved in 10 mls of sterile distilled water and then added prior to pouring into plates.
    - The solution is poured into 4 inch plates and allowed to cool for 24 hours.
    - After 24 hours, the plates are placed in cold storage at 4 degrees Celsius.

3.5 *Hay Analysis*

- Five core samples are taken from the short end of a hay bale prior to steaming and an additional five core samples were taken from the opposite short end of the bale after steaming. Samples were taken from a total of 6 bales pre- and post-steaming. A diagram of the location of the core samples is below.

- The five core samples are mixed together to form one sample.
- The sample is divided in half. One half is frozen for nutritional analysis and the other half is used to fungal and bacterial culture.
- All cultures are performed in duplicate using a wet and dry methodology.
    - For the dry preparations, two quantities (0.05 g and 0.10 g) of hay are applied directly to the media.
    - For the wet preparations, 7.5 grams of hay is added to 375 ml of distilled water to create a 2% (weight to volume) solution which is considered the 1X concentration. Serial dilutions are performed to yield 1:10X and 1:100X concentrations. One hundred microliters of each concentration are applied to the media.
- Once the samples are applied to the plates, the plates are cultured at room temperature.
- Bacterial and fungal growth is counted as colony forming units (CFUs) daily for a total of 5 days.
    - Bacterial CFUs are determined based on growth on PDA only since *Aspergillus spp.* and *Fusarium spp.* selective media contained antibiotics.
    - Fungal CFUs are counted on all media.
- If a plate reaches greater than 75 CFUs, the number of colonies are deemed too numerous to count and the plate is discarded.
- The number of colonies are then determined by going to the next lower concentration (0.05 g for the dry preparation and 1:10X for the wet preparation) and the number of CFUs are multiplied by 2 and 10 respectively to allow statistical analysis of the number of colonies based on 0.10 grams dry preparation and 100 ul wet preparation (CFU/0.1 grams hay and CFU/100 ul of a 2% solution).

REFERENCES

1. Gerber, V., et al., *Airway mucus in recurrent airway obstruction--short-term response to environmental challenge.* J Vet Intern Med, 2004. **18**(1): p. 92-7.

CHAPTER 4: ADDITIONAL EXPERIMENTS AND RESULTS NOT INCLUDED IN CHAPTER 2

4.1 *Hay Analysis*

Samples from 6 hay bales were collected for analysis. Five core samples were taken from one end of each square bale prior to steaming and an additional five core samples were taken from the opposite end of the square bale post-steaming to 160 degrees Fahrenheit. Each set of 5 core samples were combined to form one sample. The sample was then divided in half with one half used for fungal and bacterial culture and the second half was frozen until it was submitted for nutritional analysis that was performed by Equi-analytical in Ithaca, New York. Statistical analysis was performed using SAS® software and included a paired t-test and Wilcoxon signed rank test. Significance was set at a p-value $\leq$ 0.05.

The mean percent moisture increased from 9.8% pre-steaming to 25.9% post-steaming ($p<0.0001$) and subsequently, the mean dry matter percentage decreased from 90.2% pre-steaming to 74.1% post-steaming ($p<0.0001$). All remaining nutritional analysis was performed on a dry matter basis.

Digestible energy (Mcal/lb of sample) decreased from 1.087 to 1.037 post-steaming ($p=0.0199$). Steaming increased crude protein percentage (19.73% to 20.47%, $p=0.0354$), estimated lysine percentage (0.927% to 0.958%, $p=0.0323$), ADICP percentage (1.33% to 2.73%, $p=0.0062$), and Neutral Detergent Fiber (NDF) percentage (44.12% to 47.93%, $p=0.0234$). On a g/dl basis, steaming increased crude protein (89.65 g/dl to 92.85 g/dl, $p=0.0438$), ADICP (6.10 g/dl to 12.37 g/dl, $p=0.0059$), and NDF (200.2 g/dl to 217.5 g/dl, $p=0.0246$). Steaming tended to increase estimated lysine in g/dl. Steaming decreased Non-Fiber Carbohydrates (NFC) percentage (25.22% to 20.68%, $p=0.0184$) and g/dl (114.3 g/dl to 93.8 g/dl, $p=0.0179$).

Steaming decreased potassium percentage (2.54% to 2.35%, $p=0.0375$) and potassium g/dl (11.5 to 10.6, $p=0.0387$). Steaming decreased sodium percentage (0.024% to 0.0148%, $p=0.0012$) and g/dl (0.108 g/dl to 0.067 g/dl, $p=0.0016$). Steaming tended to decrease calcium (percentage and g/dl) and zinc (ppm and g/dl). Steaming decreased molybdenum from 0.933 ppm to 0.567 ppm ($p=0.0197$) and 0.433 g/dl to 0.250 g/dl ($p=0.0284$).

Alpha tocopherol (vitamin E) was increased (8.14 IU/lb to 11.88 IU/lb, p=0.0378) and tended to increase beta-carotene (vitamin A). Reported levels were too low to detect differences in retinol (vitamin A) and ergocalciferol (vitamin D).

Steaming significantly reduced the relative feed value (RFV) from 130.3 to 116.3 (p=0.0443), however the score for the hay remained above the reference value of 100.

There was no statistical difference between pre- and post-steaming samples in percentage (%) or g/dl of Acid Detergent Fiber (ADF), Water Soluble Carbohydrates (WSC), Simple Sugars (ESC), starch, phosphorus, and magnesium. There was also no difference in parts per million (ppm) or percentage (%) of iron, copper, and manganese.

4.2 *Mineral, Feed and Water Intake*

Mineral intake tended to be greater in horses fed steamed hay (1.5 ounces/day) than dry hay (0.7 ounces/day) (p=0.0992). Hay and water intake as well as changes in body weight were discussed in chapter 2. There was no difference in body condition scores over the 10 day feeding period or between groups.

4.3 *Discussion*

While the structural components of the hay were not analyzed, the changes in crude protein, ADICP and NFC are most likely the result of alterations in proteins during steaming. The change in protein structure combined with leaking of electrolytes during steaming likely resulted in the observed differences in electrolytes. Analysis of the water run-off from steaming would have allowed confirmation of the hypothesis that electrolytes were lost as part of the steaming process.

Horses fed steamed hay consumed less hay on a dry matter basis yet gained more weight than while they were fed dry hay. Hay analysis showed that steaming reduced the digestible energy on a Mcal/lb basis while increasing the amount of crude protein. While the amount of increased protein may

have improved weight gain, decreased energy intake should reduce body weight. It is unclear how steaming affects the digestibility of hay when it is consumed by horses.

While being fed steamed hay, horses tended to increase their intake of mineral supplement while maintaining similar water intake. It is likely that horses increased their mineral intake to compensate for the lower values in the steamed hay and that by maintaining normal blood concentrations of these electrolytes; they did not alter their water consumption. Blood concentrations of these electrolytes were not performed and would be needed to fully assess changes in mineral intake.

4.4 *Future Work*

The information presented here raises many questions which necessitate further work to answer. A digestibility trial would allow further evaluation of the nutritional changes seen in the hay post-steaming and their effect on weight gain. A longer feeding period would allow analysis of long-term changes in weight and body condition scores. Analysis of the run-off produced during steaming could confirm the suspected mechanism of electrolyte loss. In addition, the analysis of the run-off would provide guidance for supplementing horses fed steamed hay or for feeding selected groups of horses that may benefit from dietary modifications.

In direct relation to RAO-affected horses, a study with a greater number of subjects would decrease variability and increase study power enabling confirmation of the results we identified in this study. Combing hay steaming with additional environmental modifications commonly used in the management of RAO-affected horses may allow determination of the full benefit of steaming hay.