COMPARATIVE EFFICACY OF THREE COMMON TREATMENTS FOR EQUINE RECURRENT AIRWAY OBSTRUCTION.

Laura Caryn Lee

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Virginia Buechner-Maxwell
Sharon Witonsky
Kurt Zimmerman

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ABSTRACT

Objective – evaluate horses with acute airway obstruction using three treatment regimens: tapering doses of dexamethasone (DEX), environmental modification (ENV), and a combination of both treatments (DEX + ENV) by analyzing clinical parameters, pulmonary function testing, bronchoalveolar lavage fluid (BALF) cytology and BALF cell expression of the cytokines IFN-γ and IL-4

Animals – 6 horses with recurrent airway obstruction (RAO)

Procedures – Clinical examination, pulmonary function test, and collection of BALF prior to treatment and during 22 day treatment period

Hypothesis - Alterations in clinical parameters, pulmonary function and airway inflammation in acute equine RAO will return to remission values by treating with DEX, ENV or DEX + ENV

Results – All horses demonstrated clinical disease, reduced pulmonary dynamic compliance (C_{dyn}) and an increased maximum change in pleural pressures (ΔPpl_{max}) when in a challenge environment. All treatments improved clinical parameters, ΔPpl_{max} and C_{dyn}. BALF cytology during an RAO crisis demonstrated neutrophilic inflammation. ENV or DEX + ENV resulted in a significant decrease in airway neutrophilia that was maintained throughout the treatment period. In contrast, treatment with DEX caused a reduction in airway neutrophilia initially followed by a rebound neutrophilia as the period between administrations of dexamethasone (0.05mg/kg) was increased to 72 hours. The rebound neutrophilia was not accompanied by equivalent deterioration in clinical parameters or pulmonary function.

Conclusions – Environmental modification is important in the management of RAO horses. Treatment of clinical RAO with a decreasing dosage protocol of corticosteroids in the absence of environmental modification results in the persistence of airway inflammation without recrudescence of clinical disease.

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DEDICATION

To my mom, dad, brother and sister as well as my grandparents back home in South Africa. Thanks for all your support during the past few years away from home. You allowed me to follow my dreams and let them take flight. Thank you for always being there for me.

"If you can dream it…you can achieve it"

ABBREVIATIONS

BALF	Bronchoalveolar lavage
C_{dyn}	Dynamic compliance
DEX	Dexamethasone treatment
DPBS	Dilute phosphate buffered saline
ENV	Environment treatment
GC	Glucocorticoid
GC-R	Glucocorticoid receptor
GM-CSF	Granulocyte/monocyte – colony stimulating factor
ICAM-1	Intercellular adhesion molecule 1
IgE	Immunoglobulin E
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
INFγ	Interferon gamma
MSF	Monocyte stimulating factor
NFκβ	Nuclear factor kappa beta
RAO	Recurrent airway obstruction
Th1	T helper 1
Th2	T helper 2
TNF	Tumor necrosis factor
ΔPpl_{max}	Maximum change in pleural pressures

TABLE OF CONTENTS

Chapter 1

1.1 Introduction

Equine recurrent airway obstruction (RAO), formerly known as chronic obstructive pulmonary disease or heaves is a respiratory condition that typically affects horses, 7 years of age or older, whereby clinical signs develop in association with stall confinement. RAO has a seasonal occurrence which is more evident in the northern hemisphere. This finding is thought to be due to the increased period of time that the horse spends indoors during the winter months as well as inadequate drying of hay during wetter periods. This condition resembles human asthma and similarly is thought to be associated with a pulmonary hypersensitivity reaction to inhaled allergens. Clinical signs seen in horses affected with RAO vary in severity but classically are considered to involve acute episodes of airway obstruction which are reversible. Common clinical signs include coughing, exercise intolerance, nasal discharge and increased breathing effort. Remission is induced by removing the horse from the inciting agents. This most commonly involves pasture turnout. Horses susceptible to the disease are almost indistinguishable from healthy horses which are not experiencing an acute RAO crisis. Severe cases can often be diagnosed based on historical and clinical findings alone, however the analysis of bronchoalveolar lavage fluid (BALF) which characteristically demonstrates a neutrophilic leukocytosis (15-85%) [1] is considered to be a useful diagnostic aid.

Despite recognition of the disease for centuries, the pathogenesis is still not entirely understood. However, it is known that there is a complex interaction of genetic and environmental factors that interplay with the pulmonary immune system resulting in the characteristic findings of lower airway inflammation, bronchospasm, airway hyperresponsiveness and excess mucus secretion. The primary trigger is thought to be inhaled allergens such as fungal elements and endotoxins which are commonly found in poor quality hay and straw bedding. Other factors shown to be important include the stall design, ventilation, hay feeding and age of the horse. [2]

The pulmonary hypersensitivity reaction has been extensively studied. Investigating genetic markers of cytokine expression has allowed a better understanding of the immune reactions and mediators involved. The recent availability of the horse genomic sequence has allowed a familial predisposition of RAO to be proven. [3,4] Genetic factors are also associated with the severity of clinical signs, specifically coughing frequency.

The mainstay of treatment remains long term environmental modification in order to minimize exposure of the horse to inciting allergens. Intermittent anti-inflammatory corticosteroid medications and bronchodilators are often administered either systemically or via inhalation to aid in the induction of remission. Most horses respond well to the currently used treatment approaches, however the medications used are not without unwanted side effects and so preventative measures together with new treatment options are required.

Gross lesions in equine recurrent airway disease often include lack of pulmonary collapse and pulmonary emphysema with histopathological findings of chronic bronchitis/bronchiolitis. Cytology imprint smears often reveal thick mucus that forms, what are known as, Curschmann's spirals. [5] This is very similar to human asthma where overinflation of the lung parenchyma with failure to collapse is typical. Mucus plugs in the air passages, marked goblet cell hyperplasia and inflammatory cell infiltration into the airway walls are well described changes that occur in human patients.

Recurrent airway disease shares a number of similarities with human asthma and is thereby a good animal model for studying pathogenesis and progression of human asthma. This is vital in advancing our understanding of a condition which was been shown to affect up to 5.4% of Americans in 1994 with an increasing prevalence on a global scale.[6]

1.2 Significance

Equine RAO is reported to be one of the most common equine respiratory diseases affecting up to 50% of the horse population worldwide. [7] Certain populations under confinement conditions may be affected to an even greater degree. This disease is considered to be important for a number of reasons. Clinical signs demonstrated during an acute episode can result in respiratory distress, reduced feed intake and persistent coughing. The disease is also associated with potentially long term detrimental effects such as cardiac and skeletal muscle pathology [8,9] as well as fibrous tissue changes within the pulmonary airways. Sport horses are of particular concern due to impaired performance during an acute episode resulting from airway inflammation and impaired gas exchange. Lost days in work and possibly irreversible changes in the pulmonary airways and skeletal muscles may also limit long term athletic career of horses. In addition, the long term cost of managing these horses, coupled with the environmental restrictions associated with disease may tax the owner's ability to keep such a horse. Treatment is effective in inducing remission in the majority of horses and the disease can be appropriately managed but requires owner compliance. As methods become available for identifying heritable characteristics associated with an increased risk of developing RAO, genetic screening may become a significant factor in parental selection. [10] Another concern in horses affected with RAO is the possibility of increased susceptibility to respiratory pathogens due to reduced mucociliary clearance and persistent inflammation.

1.3 Pathophysiology

Aristotle in 333BC described "heartache in horses" which is thought to possibly represent the first description of heaves in horses. Since that time there has been extensive investigation into the underlying pathophysiology of RAO in horses. Horses affected with RAO typically demonstrate an increased accumulation of tracheal mucus, decreased arterial partial pressure of oxygen, bronchospasm with smooth muscle remodeling and airway inflammation. This is most evident by an increased BALF neutrophil count with increased cell activation. The exact

pathogenesis of these findings remains uncertain despite the large body of research into this topic. The majority of evidence does point towards a modified pulmonary inflammatory response to inhaled allergens in affected horses.

Yu and coworkers demonstrated that there were a number of mediators involved in the pathogenesis of RAO. Studies demonstrated that horses with heaves had dysfunction of the inhibitory non-adrenergic-non-cholinergic receptors, diminished response of the smooth muscle to cholinergic activation, and enhanced function of epithelial derived relaxing factor. The inhibitory function of prostanoids was also reduced resulting in a change in prostaglandin production in favor of excitatory prostanoids. [11]

Humans with asthma have been shown to demonstrate an early phase response as a result of inhaled organic dust. This response is seen by the onset of signs of airway obstruction when exposed to inhaled dust. This is typically seen as coughing, wheezing, shortness of breath and chest tightness. Healthy horses typically demonstrate a mild response when dust is inhaled while an early response is absent in horses with RAO. This suggests that horses with RAO may have a compromised early phase response to inhaled allergens. This response may account for the signs seen where horses with RAO may take a number of days to demonstrate signs of airway obstruction when placed into a challenge environment. [12]

1.3.1 Environment

The horse's environment plays a very important role in the pathogenesis of RAO. Early studies demonstrated the clear association with RAO-susceptible horses and their environment, specifically dusty stalls and hay feeding. Consequently, many researchers have concentrated their efforts on environmental factors for the purpose of treatment and prevention of clinical disease in affected horses. The typical response of RAO-horses to a challenge environment includes coughing, increased breathing effort and nasal flaring along with changes in pulmonary mechanics and the development of airway hyperreactivity. Inflammatory cell recruitment and activation within the lungs following exposure of susceptible horses to allergens is typical of RAO. A seasonal pattern of clinical exacerbation has been noted and associated with increased environmental temperatures and humidity, increased fungal spore counts and management changes such as increased time in the stall and hay feeding.

The association between clinical signs of RAO and exposure to aerosolized debris has been further substantiated in studies that compare susceptible horses to healthy horses when housed on pasture and in a challenge environment. In the pasture environment, the healthy horses and those affected with RAO demonstrated similar clinical and functional pulmonary parameters including bronchial reactivity. RAO affected horses were then moved to stall confinement and fed only hay silage which had previously been shown not to contain many aeroallergens.[13] The RAO horses demonstrated an intermediate airway reactivity compared to horses at pasture or in RAO horses in an acute crisis.[14] In addition, lung function of RAO horses experiencing acute, severe

disease improved after 6 hours [15] of environmental modification and returned to clinical remission when horses were placed in a pasture environment. [16-19]

Many horses are stalled for large portions of the day where they are exposed to the stall environment and its associated allergens. Studies have compared the effects of stall design as well as constituents of the stall environment such as bedding and hay feeding as important sources of allergens. These studies provide evidence that changes in feed, bedding and other modifications can significantly influence the level of debris to which the horses are exposed.

a) Air hygiene

Air hygiene in the stall affects the incidence and severity of airway disease in horses. [20,21] Ventilation is considered to be the most important factor in the removal of airborne particles and the delivery of clean air. Good air quality is dependent on the air changes per hour as well as the air space provided per horse. Equilibrium exists between the dispersal and deposition of airborne particles in the air. The concentration and rate at which the equilibrium is reached is significant. Therefore minimizing total airborne particles and increasing the rate of clearance are recommended. Dilution by ventilation is one of the major mechanisms of airborne particle clearance. [22] When stall design was assessed, investigators found that free-standing box stalls were more likely to have adequate ventilation when compared to a barn-type set-up. Standard mucking out techniques doubled the number of airborne particles in a barn with good ventilation and increased the concentration up to 16.8 fold in a barn with poor ventilation. [23]

Airborne particles in the horse's environment are composed of dust; dust mites; mold and fungal spores. The airborne particles are inhaled by the horse and move from the nares into the airways where the particle size determines the depth of penetration into the airways. Larger heavier particles sediment out earliest and are removed via mucociliary clearance in the airways. The smaller particles have the potential of reaching deeper into the airways. Soluble particles can move into the bronchial circulation or lymphatic system. Pathogens at the alveolar level are cleared by alveolar macrophages. Factors such as travel, training or strenuous exercise have been shown to affect the phagocytic ability of the alveolar macrophages. [24,25]

b) Mold

Specific agents such as molds and mite dust were suspected to be important in the pathogenesis of RAO as these agents have been shown to be important in humans with asthma. The molds *Faenia rectivirgula* (*Micropolyspora faeni*), *Aspergillus fumigatus* and *Thermactinomyces vulgaris* [26,27] were found to be most important. However, respiratory challenge with these agents induced a less marked alteration in respiratory function of RAO-affected horses and was therefore considered to be less specific than a natural challenge. [28] Hay dust, endotoxin and the molds, *A. fumigatus* [26] and *F. rectivirgula* have been most extensively studied as suspected airway allergens found in the horse's environment. Small fungal or mold spores can take a long time to settle out of the air and remain allergenic even when no longer viable. Challenge with *A.*

fumigatus in RAO susceptible horses resulted in an increased total cell count in BALF and an increase in macrophage and lymphocyte expression of IL-1β and IL-8. [29] Aerosolized exposure to an Aspergillus extract produced a significant increase in neutrophil counts in the BALF of horses exposed to this agent verse those horses not exposed to this agent. More significant lung dysfunction was noted with higher doses. These findings support the idea that *A. fumigatus* plays a role in RAO, however there are other components which also are involved. [30]

F. rectivirgula is also thought to be one of the more important airborne molds .[27] When administered via aerosol challenge, healthy horses showed no effect on pulmonary mechanics or gas exchange. In contrast, RAO affected horses experience a significant increase in respiratory frequency and minute ventilation with a decrease in the arterial partial pressure of carbon dioxide. *F. rectivirgula* exposure also was associated with an increase in the total cell count, number of neutrophils and albumin concentration in BALF samples of both healthy and RAO affected horses. Airway responsiveness was shown not to be affected. [31] Specific types of fungal spores such as Basidospore, Nigrospora and Curvularia spp. [32] have also been associated with RAO.

The research of Madelin and coworkers demonstrated that there were significantly more precipitating antibodies to mold in the sera of RAO horses stalled in a barn verse sera from horses housed in free standing stall. However serum precipitins antibodies to mold antigens have been demonstrated in clinically normal horses. Serological tests are therefore considered to be of minimal use as a diagnostic tool in diagnosing RAO. [33]

c) Endotoxin

Endotoxin in the hay is suspected to play a role in RAO because it is a strong stimulator of the immune response. When horses were exposed to aerosolized endotoxin, both healthy and RAO-affected animals developed a dose-dependent airway neutrophilia consistent with airway inflammation. Yet, endotoxin inhalation failed to change the clinical score, airway reactivity or mucus accumulation in either the RAO affected or healthy horses. [34,35] In a second study, the response of horses challenged with an extract of *A. fumigatus* was compared to their response to the same extract in which all components of endotoxin were removed. A significant reduction in airway neutrophilia accompanied by an increase in arterial oxygen tension was observed in horses which received aerosolized, endotoxin-free extract as compared to the response of horses receiving extract including endotoxin. The response to the endotoxin-free extract was also considered similar to horses not exposed to the mold at all. The cytokine profile in peripheral white blood cells was evaluated in RAO-affected horses following inhalation of lipopolysaccharide. In response to this challenge, white blood cells demonstrated an increase in IL-10, IL-4 and TNF-α expression. [36] These studies support the idea that although it is not the only determinant of disease severity, endotoxin contributes to the pathogenesis of RAO. In RAO-affected horses, endotoxin appears to amplify airway response to other aerosolized debris like fungal molds. In normal horses, exposure also causes airway inflammation but does not

initiate the sequence of events that result in severe respiratory compromise observed in RAO affected horses.

d) Hay

Feeding hay has long been thought to be associated with RAO in horses. [37] Horses with heaves respond to a hay dust challenge by developing the typical signs of RAO including airway neutrophilia, obstructive airway dysfunction and airway mucus accumulation while healthy horses subjected to the same study demonstrate airway neutrophilia with no airway obstruction or mucus accumulation. [38] The response in RAO horses was more substantial. The authors then evaluated the different components of hay dust solution and found that dust particulates do contribute to the pulmonary recruitment of neutrophils in RAO. [39]

The association between hay exposure and disease has also stimulated studies aimed at examining factors that influence hay dust content, such as conditions at the time of baling. Hay baled with high moisture content contains an increased amount of molds such as *A.fumigatus, F. rectivirgula* and *T.vulgaris.* The way in which hay is fed (off the ground verses in a rack) also influences level of exposure, especially if the horse's face is in close contact with the hay for large portions of the day. These factors impact the severity of response.

e) Bedding materials

Bedding is another principal part of the stall environment and its role in RAO has evaluated. Bedding materials were compared for concentration of dust in the stall environment as well as the amount of mold associated with each type. Natural bedding is commonly used in horse stalls and so wood shavings, paper and straw were compared. Other synthetic materials have been shown to potentially have a lower concentration of dust however the increased risk of intestinal obstructions limits their use. Wood shavings are a common natural bedding material. They were found to be more resistant to molding than other plant based materials such as paper. [20] Wyse and coworkers compared the use of straw bedding and the feeding of hay with a reduced dust regimen consisting of paper bedding and feeding ensiled grass. [40] The RAO-susceptible horses housed on the reduced dust regimen showed fewer clinical signs of airway inflammation. Deep litter management increased molding. Factors such as humidity, temperature and time since bedding are also important.

Woods and coworkers compared the use of management system using hay feeding and straw bedding to that using wood shavings and a complete pelleted feed in terms of airborne dust concentrations. The system using hay feeding and straw bedding had significantly higher total respirable dust concentrations in the stall with higher concentrations in the breathing zone of the horse than in the stall. The pelleted feed and wood shaving bedding system produced only 3 % of the respirable dust burden in the breathing zone of the horse when compared to the system utilizing straw bedding and hay feeding. In this study there was a higher mean total airborne dust concentration during the daytime period when compared to evening hours but no significant

difference between summer and winter. [41] Clark and coworkers found that the respirable dust did not in fact decrease at night but rather that the size of particles remaining in suspension was smaller with the heavier particles sedimenting out of suspension.[21]

These studies lead to the conclusion that there are combinations of factors in the environment that are capable of eliciting an RAO response in affected horses.

1.3.2 Smooth muscle remodeling and pulmonary fibrosis

An increase in airway smooth muscle mass has been shown to be a feature of asthmatic airway remodeling in both humans as well as in rodent models of asthma.[42] Smooth muscle from chronically affected RAO horses demonstrates cell proliferation as well as the presence of apoptotic cells. The increased myocyte proliferation was associated with a significant increase in the amount of smooth muscle in the airways of affected horses. An increase in myocyte apoptosis was thought to represent a compensatory mechanism to limit abnormal smooth muscle growth. Airway smooth muscle remodeling may also be involved in airway hyperresponsiveness and chronic functional impairment .[43] Transforming growth factor beta 1 (TGF-β1) is a potent profibrotic cytokine that is thought to contribute to airway wall thickening and fibrosis of bronchi and alveoli submucosa in humans. However the amount of TGF-β1 found in the BALF from RAO affected horses does not differ from normal horses, even after antigen challenge. These findings suggest that while TGF-β1 is present in BALF that it does not have a causal relationship with airway smooth muscle remodeling observed in RAO affected horses. [44]

Alveolar fibrosis [45,46] has also been described in chronically affected RAO horses. The elastic properties of the pulmonary parenchyma can be altered to the extent that chronically affected RAO horses may not completely return to normal following treatment or may have a poor response to bronchodilators. Protease release from activated inflammatory cells such as neutrophils is potentially involved.[45]

1.3.3 Mucus accumulation

Horses have two types of mucus gland acini with a frequency of gland opening similar to other large mammals. Mucus hyper-production is observed in humans with asthma or chronic obstructive pulmonary disease and is associated with a number of mechanisms. These include increased activation of the calcium activated chloride channel 1(CLCA1), epidermal growth factor receptor (EGFR), mucin gene (MUC5AC), B cell lymphoma 2(Bcl-2), IL-13 and interferon-γ. Clinically affected RAO horses typically also demonstrate an increased accumulation of mucus within the airways when compared to healthy horses. The gland volume in healthy horses has however been shown to be only 15% that of other species and therefore increased mucus accumulation is not related to a species variation. Mucus plugging in RAO affected horses can lead to intractable airway obstruction .[46] Endoscopy has been used to evaluate the airways and to characterize the appearance and volume of mucus present in the airways of RAO affected horses. A system has been created to standardize endoscopic grading of

mucus accumulation. The mucus grade scale increases in scale from one to five as the amount and location of the mucus in the trachea changes. RAO-affected horses were shown by Gerber and coworkers to typically have a mucus score of 4-5 while housed in a challenge environment (stall with straw bedding and fed dusty hay) compared to healthy control horses in the same environment which had a large variation in response with an average score of about 2-3.[47]

Goblet cell metaplasia in bronchioles and goblet cell hypersecretion in the bronchi and trachea of clinically affected RAO horses has been demonstrated in the absence of an increase in the number of mucus-gland associated cells. [48] The rheological properties of mucus in clinically affected RAO horses increased viscoelasticity, a decreased mucociliary clearability index and decreased cough clearability index compared to normal horses or RAO horses in remission. [49] These changes may contribute to the mucus stasis and accumulation typically seen in horses affected with airway obstruction. Airway inflammation has been correlated with the mucus cell type and the amount of stored mucosubstances. While therapy to decrease airway inflammation has been shown to decrease mucus accumulation ,[50] changes in rheological properties do not correlate with BALF cytology. [49]

Two main types of mucin genes have been identified in horses – equine mucin gene 5AC (eqMUC5AC) and equine mucin gene 2 (eqMUC2). Gerber and coworkers reported that the eqMUC5AC gene is expressed in the glandular stomach, colon and all airways while the eqMUC2 gene was expressed in the colon exclusively.[47] In horses with RAO, expression of eqMUC5AC is upregulated and may contribute to mucus accumulation observed in horses with this disease.

Jefcoat and coworkers provided evidence that RAO-affected horses experiencing an acute episode of airway obstruction had increased levels of 4E4-immunoreactive glycoprotein (E4E) (a carbohydrate side chain specific monoclonal antibody, as well as increased levels of mucin associated carbohydrates fucose(α-1.2 linkage) and N-acetylglucosamine. Horses in remission continued to have elevated 1, 2 fucose and N-acetylglucosamine with a trend towards increased 4E4 levels above controls. This evidence supports that there are persistent changes in quantity and quality of mucus glycoproteins in RAO horses. [51]

While it is unclear whether the volume of secretions, rheological properties or reduced clearance play the most significant role in mucus accumulation in the airways of clinically affected RAO horses it is likely that they all contribute.

1.3.4 Beta-adrenergic receptors in RAO

The β2 adrenoreceptor (β2-AR) is a well characterized G protein-coupled receptor that is widely distributed in airway smooth muscle. [52] Activation of these receptors relaxes the airway smooth muscle, stimulates mucus secretion and ciliary function. Agonist binging of β2-AR promotes relaxation of airway smooth muscle by stimulating a cascade of events that are initiated through a conformational shift in the receptor such that the G(S) subunit is freed to bind to guanosine-5'-

triphosphate (GTP) and activate adenylyl cyclase. [53] Horses affected by RAO have been shown to have a decreased β2-AR density in the lung parenchyma (33%) and bronchial tissues (42%). Specifically, airway smooth muscle from RAO horses have a decrease in β2 subtype receptors associated with an attenuated coupling efficiency of the receptors to stimulatory G(S) protein and a decrease activation of adenylyl cyclase. [2] While density of receptors may differ between RAO and normal horses, β2 receptors are functional in RAO horses. [54] Scott and coworkers determined that both β1 and β2 receptors exist in the airways of RAO affected horses and these receptors are activated during an acute RAO episode.[55] The concentration of β1 receptors is considered to be substantially less than the β2 receptors. The predominant receptor therefore targeted in airway relaxation is the β2 receptor. Bronchodilation of the airways of RAO affected horses that occurs in response to β-2 agonist medications is considered to be primarily mediated by the β2-AR. By chemically blocking the β receptors of healthy horses resulted in no change in airway responsiveness, dynamic compliance or pulmonary resistance. The same procedure performed in clinically affected RAO resulted in increased pulmonary resistance with no change in airway responsiveness or dynamic compliance. This led the authors to believe that the beta-adrenergic system was involved in the control of airway diameter but not in mechanisms of hyperresponsiveness. [56]

1.3.5 Alpha-adrenergic receptors in RAO

In the airways the alpha-receptors have effects on mucus secretion and on the release of neurotransmitters. In the equine airways activation of the alpha-2 adrenoreceptors potently inhibits the release of acetyl choline (Ach) from parasympathetic nerves. Inhibition of Ach results in bronchodilation of the airways. It has been suggested that there is a pre-junctional alpha-2 receptor dysfunction in horses with RAO. Zhang and coworkers showed that alpha-2 receptors are dysfunctional in the airways of RAO horses by using the physiological agonists - epinephrine and norepinephrine. Healthy horses had a markedly higher response to circulating catecholamines than in RAO horses suggesting that these receptors are dysfunctional in RAO affected horses. [54]

1.3.6 Muscarinic acetylcholine receptors

The predominant excitatory innervations of equine airways are cholinergic and the bronchospasm characteristic of RAO has been attributed to enhanced activity of these nerves. Horses affected with RAO do not have a significant alteration in acetylcholine receptor density, subtype distribution or number of muscarinic acetylcholine receptor binding sites. Abraham and coworkers showed no change in the expression or function of the muscarinic acetylcholine receptors in horses with RAO [57,58] while Zhang and coworkers suspected muscarinic receptor dysfunction in horses affected with RAO. [54] This was shown by adding atropine to a β-2 agonist protocol and noting that the addition of atropine did not augment the release of acetylcholine when compared to the use of β-2 agonists alone. The magnitude of augmentation in horses affected with RAO in the absence of atropine was similar to the response in control horses in the

presence of atropine. Ipratropium bromide is an anticholinergic agent that has been shown to decrease the maximum change in pleural pressures during tidal breathing as well as reduce pulmonary resistance. The duration of effect was 4-6 hours. [19] Inhibition of cyclo-oxygenase or prostaglandin E_2 (PGE_2) activity has shown no effect on acetylcholine release. [58]

1.3.7 Oxidative stress

Oxidative stress has been shown to occur in human asthma and there is also clear evidence that oxidative damage does occur in horses with RAO. Oxidative stress occurs when antioxidant defense mechanisms are overwhelmed by free radicals leading to DNA and cellular damage. Horses have an increased non-enzymatic antioxidant capacity in the pulmonary epithelial lining fluid compared to humans. This is due to the horse's ability to synthesize a higher concentration of ascorbic acid [59] and suggests that a higher oxidative load would be required to cause oxidative stress in equine tissues. In RAO affected horses, studies have shown that acute neutrophilic inflammation does not induce a marked oxidative stress response in the lungs. However prolonged inflammation may result in depletion of antioxidants and oxidative damage. The research of Deaton and coworkers indicates that RAO affected horses generally have less ascorbic acid in the tracheal epithelial lining as compared to normal horses and that ascorbic acid levels are further reduced when RAO horses experience airway inflammation. [60] In addition there is an inverse relationship between the number of neutrophils in the BALF and the ascorbic acid concentration in the pulmonary epithelial lining fluid (PELF) following challenge. The decreased ascorbic acid also correlates with an increase in pulmonary resistance. Based on these findings Deaton and coworkers conclude that oxidative stress does not occur in horses with acute airway inflammation despite a reduction in antioxidants but rather that chronic depletion of these antioxidants is necessary to cause oxidative damage. [61,62]

Pulmonary epithelial fluid glutathione status is significantly different in horses with acute airway obstruction when compared to healthy horses. [63] During an RAO crisis oxidized glutathione and glutathione redox ratio is significantly increased compared to those horses in remission. These findings confirm that oxidative stress does occur in RAO affected horses in crisis. Other researchers have also reported a significant effect of heaves on oxidant markers such as reduced and oxidized glutathione, glutathione redox ratio, uric acid, 8-epi-$PGF_{2\alpha}$ [64], catalase, superoxide dismutase and glutathione peroxidase. [65] In comparison with polymorphonuclear leukocytes from the blood of healthy horses the reduction mechanisms in BALF were faster. Kirschvink and coworkers demonstrated increased isoprostane levels in PELF of clinically affected RAO horses while there was no evidence of increased levels in the plasma. Isoprostanes are markers of oxidative stress.[66] Oxidative DNA damage in equine peripheral blood mononuclear cells is significantly greater in RAO horses in remission when compared to healthy horses.[67]

The discovery of an association between RAO and oxidative stress has lead to investigation into the clinical benefits of antioxidative supplementation. Reactive oxygen free radicals modulate the activation of transcription factors such as nuclear factor-$_{\kappa B}$ and activator protein-1 in several

cells which then influence the expression of pro-inflammatory cytokines including IL-1β. It is possible to reduce the hyperresponsiveness of the airways and the IL-1β mRNA expression in RAO crisis by pre-treating with reduced glutathione, thereby confirming the role of oxidative stress in the development of signs associated with acute RAO. [68]

High neutrophil cell accumulation results in the increased production of reactive oxygen species, one of which is hydrogen peroxide. High airway concentrations of hydrogen peroxide cause airway smooth muscle contraction by acting on muscarinic neurotransmission. Some of these effects are mediated by cyclo-oxygenase products. Hydrogen peroxide has no effect on beta-adrenergic or inhibitory non-adrenergic non cholinergic (iNANC) induced relaxation. [69]

1.3.8 Equine Calcium activated chlorine channels (eCLCA) in the small airways

Calcium activated chlorine channels (CLCA) have been identified in humans as a promising therapeutic target in asthma. Likewise, it is thought that this protein is involved in RAO in horses and that it may play a vital role in the pathogenesis of the disease by modulating the hydration of airway mucins. Equine CLCA1 is a polypeptide made up of 913 amino acids which forms a 120-kDa transmembrane glycoprotein that is processed into two cell-surface-associated subunits. The site of precursor cleavage has not been determined. Equine CLCA1 is present in mucus producing cells in the respiratory and intestinal tracts, sweat glands and renal mucus glands. RAO-affected horses have an increased amount of eCLCA1 in the lungs compared to normal controls. [70] The increase in eCLCA1 in RAO horses is linked to the increased numbers of goblet cells observed in airway tissues from these horses rather than transcriptional upregulation of the eCLCA1 gene. [71]

1.3.9 Endothelin 1

Endothelin –1 has been investigated in human asthma and thought to have bronchoconstrictive effects. In RAO horses the systemic blood levels of ET-1 are significantly increased when compared to healthy horses. RAO horses also have a negative arterio-venous ET-1 difference indicating that there is a net uptake of ET-1 in the lung. ET-1 is also found in increased quantities in BALF samples from RAO horses during an acute crisis. The levels of ET-1 in the epithelial lining fluid correlates with the reduction in lung function of RAO-affected horses suggesting that endothelin may contribute to the pathogenesis of RAO. [72] Functional studies demonstrated that endothelin-1 is a potent spasmogen of the third generation pulmonary artery (ET_A receptor) and bronchus (ET_A and ET_B receptors).[73] The distribution of ET_B receptors is significantly greater in the lungs of RAO-affected horses when compared to controls. [74]

1.3.10 Surfactant and RAO

Christmann and coworkers demonstrated that the phospholipid content of surfactant from RAO-affected horses is significantly decreased as compared to normal horses. RAO horses also had a

lower percentage of phosphatidylglycerol. In contrast the ratio of the percentages of phosphatidylcholine to phosphatidylglycerol was significantly higher than in healthy horses. [75] These abnormalities are similar to those detected in surfactant from human asthmatics. However, abnormalities (such as bronchoconstriction and mucus accumulation) observed in RAO affected horses is not known.

1.4 Immune response to inhaled allergens

When the horse's airways are exposed to potential inhaled allergens such as *A. fumigatus*, hay dust or lipopolysaccharide the size of the particle determines the depth of penetration into the lower airways. Larger particles tend to get caught in mucus in the upper airways and are moved out of the airways by the mucociliary clearance mechanisms. The cough response also assists in expelling inhaled particles. Smaller particles can penetrate further down into the smaller airways. Sedimentation occurs in the airways depending on the particle diameter with particles less than 5μm travelling deepest into the lungs. These tiny particles can be dissolved and engulfed by alveolar macrophages within the lungs *(see figure 1)* or cleared by local immune responses. Alveolar macrophages are important for the clearance of foreign material and pathogens from the alveoli and are play a vital role in the pulmonary innate immune response. Strenuous exercise or transportation is shown to decrease the number of active phagocytic cells as well as decrease the phagocytic and bactericidal activity of these cells. [18,76-78]

Once engulfed by alveolar macrophages or dendritic cells the foreign particle is processed and the antigenic protein expressed on the cell surface. Lymphocytes are specific white blood cells that are part of the acquired immune system. Lymphocytes are divided into B and T lymphocytes depending on their tissue of origin. Lymphocytes possess specific cell receptors that only recognize foreign particles once they have been processed and presented as part of the major histocompatibility complex II (MHCII) on the surface of an antigen presenting cell such as a dendritic cell. The lymphocytes then determine whether a reaction is mounted to the foreign particle or whether tolerance to the antigen exists. Once presented with antigen the T lymphocytes differentiate into various subpopulations that are distinguished by the cytokines that they produce and which are responsible for specific roles within the immune response *(see figure 2)*. Within the T cell subpopulations there is a subtype of lymphocytes known as T helper cells. These cells are important in activating and directing other immune cells. T-helper cells 0 (Th0) secrete interleukin-2 (IL-2), interleukin-4 (IL-4), and interferon gamma (IFNγ) and then differentiate into T-helper 1 (Th1) or T-helper 2 cells (Th2) depending on the predominant cytokine environment. These cytokines are specialized chemical mediators which act as signaling molecules. It is thought that cytokines and chemokines play a central role in the development and persistence of lower airway inflammation. A Th1 response is typically associated with a cellular immune response and the secretion of IFNγ and TNFα. IL-10 and IL-4 inhibit Th1 production. The secretion of IFNγ increases the production of IL-12 by dendritic cells and macrophages to further promote a Th1 response. A Th2 response is associated with B cells and a humoral immune system and is thought to be the predominant response in human

asthma. The cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 are linked with this reaction. IL-4 is important in driving Th2 cell production while IFNγ inhibits Th2 cells. The cytokine IL-2 is released by most cells and acts in an autocrine fashion to activate the T lymphocyte proliferation pathways. This simplistic T helper cell model has however been questioned with many cytokines also expressed by other immune cells and other cells expressing cytokines from both profiles.

Human asthma is a chronic allergic condition which has been associated with immunoglobulin E (IgE) and cytokine expression associated with Th2 lymphocytes. IgE synthesis is orchestrated by production of IL-4 and eosinophilic inflammation through the production of IL-5 [79-81]. In human asthma the role of eosinophils is however questioned as the administration of monoclonal antibodies against IL-5 and IL-12 decreased BALF eosinophilia without evidence of clinical improvement.

RAO affected horses have been extensively studied both during acute crisis and remission to determine the predominant cytokine profile. A complete profile of the genes related to RAO in horses could not be obtained using a human microarray. [82]

The cytokine data relating to the phenotypes of T lymphocytes in peripheral blood and BALF in RAO affected horses is contradictory. Timing of sample collection and stage of clinical disease may be explanations for the discrepancies found. [83] No differences were found between cytokine mRNA compared to protein levels and a complementary relationship between directly measured cytokine levels using immunohistochemistry or indirectly measured cytokines using polymerase chain reaction (PCR) methods.

Comparing the cytokine profile of BALF collected from RAO and healthy horses at pasture there were no differences in the majority of cytokines. However, decreased levels of IL-13 expression were however noted in RAO horses in remission. [84] Following placement of RAO horses in a challenge environment however, a number of authors have found varying results in cytokine responses. Significantly increased levels of TNF-γ, IL-1β and IL-8 expression by alveolar macrophages have been seen in RAO affected horses [85,86] when compared to healthy horses. TNF-γ [86] and IL-1β are considered to be pro-inflammatory cytokines. Increased IL-8 expression has specifically been shown in epithelial and BALF cells from RAO affected horses together with and increased immunoreactivity of IL-8 in epithelial cells. The cytokines IFNγ and IL-8 are expressed to a greater degree in BALF of chronically affected RAO horses when compared to healthy horses. [87]

IL-8 is a chemoattractant and activator of neutrophils [88] and may play a role in the increased accumulation of neutrophils in the airways of RAO affected horses during a crisis period. IL-8 is upregulated in endobronchial biopsy samples and in BALF collected from RAO affected horses exposed to a challenge environment. Researchers have shown that the increased expression of IL-8 in BALF cells was positively correlated with the relative expression of IL-6 in the BALF cells as well as with the expression of IL-10 and TGFβ in the bronchial biopsy samples. The

expression of IL-5, IL-6, IL-10, IL-17 and TGFβ in bronchial epithelial biopsies was not the same as the expression of these cytokines in BALF collected from the same RAO affected horses. [89] Contrary to these findings, Ainsworth and coworkers showed that there was no difference in cytokines between healthy horses and RAO horses in acute crisis. [87] There is a lack of significant changes in IL-6, IL-10 and TNFα mRNA in RAO crisis. [89] IL-6 is considered to be an anti-inflammatory cytokine and was found to be higher in healthy horses 6 hours following challenge compared to in RAO affected horses. [90] No change in the expression of chemokine (C-X-C motif) ligand 1 (CXCL1), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (GCSF) or toll-like receptor-4 (TLR-4) in epithelial cells from RAO horses following challenge have also been described. [91] This was supported by Kleiber and coworkers who demonstrated no significant effect on peripheral blood lymphocyte cytokine expression or BALF lymphocyte cytokine expression in RAO affected or healthy horses when placed in a challenge environment. The authors therefore concluded that there was not a specific Th1 or Th2 response but rather a general down regulation of measured cytokines in both the peripheral blood and BALF lymphocytes. [92]

Over expression of IL-4 and IL-5 mRNA by lymphocytes in RAO affected horses placed in a challenge environment was considered by the authors to suggest that a Th2 response predominates in RAO affected horses during crisis.[86,93,94] The levels of IL-4 and IFNγ expression were shown to increase at various times in the progression of RAO. [86] The expression of IFNγ has however also been shown to be decreased in RAO affected horses when compared to healthy horses in a challenge environment. [94]

IL-17 is responsible for neutrophil chemotaxis, activation and maturation and could therefore be an important cytokine in the increased neutrophilic inflammation demonstrated in clinically affected RAO horses. IL-17 is downregulated in the BALF of healthy horses placed in a challenge environment while upregulated in RAO affected horses in the same environment. [84,95]

BALF pulmonary mononuclear cells exposed to hay dust solution in vivo was shown by Ainsworth and coworkers to cause an increased expression of IL-23, IL-8 and IL-1β in both healthy horses and those affected with RAO following exposure to a challenge environment for 14 days. RAO affected horse's BALF pulmonary mononuclear cells also demonstrated an increased early expression of IL-17 and CXCL2. When exposed to lipopolysaccharide the BALF pulmonary mononuclear cells showed a similar increased expression of IL-23 and IL-8 in BALF samples from both healthy and RAO affected horses. The authors therefore concluded that the pulmonary cells collected from RAO affected horses in a challenge environment were not more responsive to the agents tested when compared to healthy horses housed in the same challenge environment. [91]

Natural challenge by inhalation results in a rapid migration of neutrophils into the airway. [96] The time period between challenge and the collection of a BALF sample with a high percentage of neutrophils is as short as 5 hours .[28] The recruitment of neutrophils may be the result of a specific

immune reaction similar to eosinophils in human asthma. [97] However some airway neutrophilia also occurs in normal horses in a dusty environment [98] which is likely due to non-specific immune mechanisms. Neutrophils produce a large number of cytokines which may play an important role in RAO. Dewachi and coworkers were able to demonstrate that there are receptors on the neutrophils found in peripheral blood for the Th2 cytokines IL-5 and IL-9. [99] When RAO susceptible horses were stalled there was an increased number of neutrophils expressing these receptors when compared to controls.[99] In RAO affected horses treated with low dose corticosteroids, clinical and functional improvement occurs with persistence of BALF neutrophilia. [93]

There is also evidence of delayed neutrophil apoptosis in horses with RAO which may contribute to the persistence of neutrophils. [100] BALF neutrophils from horses clinically affected with RAO demonstrated a significant delay in apoptosis when compared to blood neutrophils from the same horse as or BALF and blood neutrophils from healthy horses. This increased survival was thought to be due to the suppression of GM-CSF receptors by antibodies which inhibit the GM-CSF activated pathways. [100]

Horses affected with RAO have a lower combined immunoglobulin G (IgG) in BALF and serum when compared to healthy horses. [101] A difference between IgG isotypes also exists between healthy and RAO affected horses. This is consistent with RAO-affected horses mounting a weaker IgG response than healthy horses. Possible explanations for this occurrence include increased destruction of antigen prior to its interaction with the lymphocytes, down regulation of IgG produced by inhibitory cytokines or increased binding of IgGb to the Fc receptors on neutrophils present in the lung of RAO-affected horses. [101]

IgE is an antibody isotype that plays a role in type 1 hypersensitivity reactions. The high affinity receptor for IgE (Fc epsilon R1) is considered to play a central role in IgE mediated responses. Cross-linking this receptor with IgE is known to result in the activation of mast cells and basophils. The production of these IgE antibodies are regulated by the cytokines IL-4 and IL-13. The finding that allergen specific IgE is present in the BALF as well as the sera of RAO-affected horses was initially interpreted as evidence that an IgE mediated hypersensitivity reaction contributed to the pathogenesis of RAO. However based on results of more recent studies, the role of IgE has become less clear. The levels of serum IgE against mold extracts have been shown to be significantly higher in RAO affected horses when compared to healthy horses. A significantly greater number of RAO horses demonstrated an IgE response to *Aspergillus fumigatus* derived antigens than healthy horses and this response was not affected by animal age. However due to the large overlap in levels of IgE between healthy horses and those with RAO, this test is not considered to be consistently useful in the diagnosis of RAO. [102] IgE positive cells have been detected in lung tissue with no significant difference between RAO-affected and healthy horses.

1.4.1 Toll-like receptors

Stall dust has been shown to be rich in endotoxins which are typically recognized by the toll like receptor 4 (TLR4). In humans it has also been shown that stimulation of this receptor results in the increase in IL-8 mRNA expression. When RAO susceptible horses were placed in a challenge environment an increase in TLR4 was found in bronchial epithelium when compared to healthy horses and to RAO susceptible horses in remission. The increased TLR4 correlated with the IL-8 levels, which in turn correlated with the number of neutrophils in the BALF of RAO affected horses. Therefore the increased TLR4 may contribute to the increased IL-8 production leading to an exaggerated neutrophil accumulation in response to stall dust. [103]

1.4.2 Platelets

Platelet activation has been shown to occur in a number of laboratory animal models of inflammatory disease and human asthmatics. The predominant effect of platelets in lung injury is due to the release of vasoactive substances which contribute to pulmonary hypertension. Secondary effects of the released vasoactive substances include increased leukocyte adhesiveness, aggregation and chemotaxis. [104] Platelet activating factor (PAF) is a membrane derived mediator from activated cells such as platelets, alveolar macrophages and neutrophils which can initiate neutrophil chemotaxis, aggregation and degranulation. PAF has been shown to cause bronchoconstriction, vasoconstriction and microvascular permeability in response to endotoxin challenge. Platelet P-selectin is also necessary for the transmigration of inflammatory cells into the pulmonary system. Dunkel and coworkers showed that circulating platelets were activated 24hours after exposure to allergens in ponies affected with RAO. [105] Hammond and coworkers further investigated the role of platelets in RAO by evaluating plasma 5-Hydroxytrytamine (5-HT).[106] 5-HT known as serotonin is stored in platelets and released on activation to aid in vasoconstriction. RAO affected ponies have significantly increased levels of 5-HT is not further increased following antigenic stimulation. PAF-induced platelet aggregation and thromboxane also remained unchanged. [106]

1.4.3 Innervation

The equine airway, like other species, receives both cholinergic and adrenergic innervation. In addition to these classical pathways there are also inhibitory and excitatory non-adrenergic-non-cholinergic receptors (NANC) and other mediators which affect airway function. An imbalance between excitatory and inhibitory pathways is suspected to play a role in the hyperresponsiveness of the airways seen in human asthma as well as in RAO affected horses. Only excitatory innervation is present in the equine distal airways through the parasympathetic system .[107] Broadstone and coworkers showed that there were both sympathetic and NANC in the equine trachea but only NANC receptors in third generation bronchi. [108] Yu and coworkers showed that there was an NANC receptor dysfunction in RAO affected horses. [11] There was also a decreased response to cholinergic activation in those horses affected by RAO. Excitatory input from cholinergic nerves has been shown to largely determine the small airway tone with a minor

effect on pulmonary parenchyma. The parasympathetic, sympathetic and NANC nervous systems play a role in the events leading to airway constriction due to smooth muscle contraction. [11,58,107-109] Contraction of smooth muscle in the airways leads to diffuse airway obstruction. Inflammatory mediators facilitate the parasympathetic mediated bronchospasm. There is however no increase in the number of muscarinic receptors or alteration in presynaptic inhibition in RAO affected horses, nor is there a decrease in cholinesterase activity.[110,111]

1.4.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. They belong to the family of proteases and are responsible for degrading extracellular matrix, processing bioactive molecules, releasing apoptotic ligands and activating chemokines. MMPs are important in cell proliferation, migration, differentiation, angiogenesis and host defense. An increased activity of MMPs occurs in the equine respiratory tract of RAO affected horses. [112] Challenge with inhaled allergens including endotoxin and hay dust [113] induced a dose dependent increase in MMPs which included pro-MMP-9, active MMP-9, pro-MMP-2 and active MMP-2. The levels of MMP-9 were highly correlated with the BALF neutrophil counts, however no association was found between MMP-2 and BALF neutrophil counts. The level of MMP-9 can be therefore be used as an indication of airway inflammation in RAO affected horses and likely play a role in airway inflammation.

1.4.5 Metabolites of arachidonic acid

Cyclo-oxygenase products of arachidonic acid have been shown to be altered in horses with RAO. In the small airways endogenous inhibition of prostanoids modulate the contractile response to nerve stimulation. In RAO affected horses histamine release and altered prostanoid profile contribute to cholinergic mediated small airway obstruction.[111] PGE_2 is a prostaglandin produced by the arachidonic cascade that acts as a broncho-relaxant in the respiratory system. RAO affected horses produce less epithelial PGE_2 than healthy horses. The level of epithelial PGE_2 correlates with the time it takes to develop signs of airway obstruction. [114] Plasma thromboxane B_2 which is a product of the arachidonic cascade is significantly increased during an acute episode of RAO. When the non-steroidal anti-inflammatory medication, flunixin meglumine was administered to horses with clinical RAO the levels of thromboxane B_2 decreased but there was no change in the degree of airway obstruction or hyperreactivity seen. These mediators therefore do not play a role in airway obstruction or hyperreactivity of RAO affected horses. [18]

Leukotrienes are naturally produced eicosanoid lipid mediators that are important in the inflammatory response. Leukotrienes are produced from arachidonic acid by the enzyme 5-lipoxygenase. The lipoxygenase pathway is found in many leukocytes including neutrophils, eosinophils, monocytes, basophils and mast cells. Anti-leukotriene treatment is effective in human asthma and so the role of leukotrienes in RAO was investigated. Neutrophils and BALF

cells from healthy and RAO affected horses demonstrate higher leukotriene B4 (LTB4) than leukotriene C4 (LTC4) levels. The level of LTB4 produced by neutrophils from RAO horses however was less in comparison to healthy horses at all times. Within 6 hours of stabling RAO affected horses there was an increase in LTB4 which was not seen in healthy horses. This increase coincided with the migration of neutrophils into the airways and is thought to play a role in airway inflammation. [115,116] The lung parenchyma has some LTB4 hydrolase activity which enables LTB4 formation. Lavoie and coworkers evaluated the effects of an LTD4 antagonist [117] which reduces LTD4 binding and LTD4 induced bronchoconstriction .[78] The drug showed no effect on cytological findings or pulmonary function. This was thought to be because the drug may not have been well absorbed or because LTD4 may not be important in the pathogenesis of RAO.

Nuclear factor kappa-beta (NF-κB) is a protein complex that is found on almost all animal cells and acts as a transcriptional factor. NF-κB is released from the cytoplasm and migrates to the nucleus where it is responsible for controlling the initiation of transcription of the inflammatory genes. NF-κB dependent inflammatory gene expression has been shown to be persistent in the bronchi of humans affected with asthma even after the presence of the allergen is removed. In RAO affected horses the level of NF-κB in both bronchial epithelial cells and BALF cells is increased [118,119] however the prototypical p65-p50 NF-κB heterodimers is absent, replaced by mostly p65 homodimers. Initially it was postulated that not all subunits were functional, however it is now apparent that both homo- and heterodimeric complexes can bind to DNA. When investigated in horses 3 weeks following an acute RAO crisis NF-κB was shown to correlate with the total long resistance, proportion of BALF neutrophilia and degree of residual lung dysfunction present. The sustained NF-κB activation in the bronchi was found to be driven by neutrophils and mediated by IL-1β and TNFα. Specific NF-κB antagonist therapies could therefore potentially improve lung function in RAO horses based on these findings. [118]

Activator protein-1(AP-1) is a transcriptional factor, similar to NF-κB that regulates gene expression. There is no significant difference in AP-1 levels between RAO horses in remission and healthy horses. AP-1 is however increased in the airways of RAO horses during acute obstruction. A positive correlation was found between the AP-1 binding activity and the induction of airway obstruction and inflammation. The DNA binding activity of cyclic AMP response element binding protein (CREB) is significantly higher in RAO affected horses following two months on pasture when compared to healthy horses. AP-1 and CREB are considered by the investigators to play a role in modulating airway inflammation in horses with RAO. [120]

Gray et al (1992) determined that the lung is the source of the immunoreactive compound 15-hydroxyeicosatetraenoic acid (i15-HETE) and it is significantly increased in the plasma of clinically affected RAO ponies. A negative correlation exists between i15-HETE and the pulmonary dynamic compliance following exposure to a challenge environment. [121]

1.4.6 Similarities to human asthma

Equine RAO shares number of similarities with dust induced human asthma. These include airway inflammation, hyperresponsiveness, reversible airway obstruction and an increase in NF-κB expression. Mast cell degranulation is believe to act as the key event in initiating human asthma and is the airways main response to inhaled allergen challenge. [122] Histologically, lung tissue from human asthma patients is similar to the changes seen in RAO affected horses, although tissue from RAO affected horses may have more changes associated with inflammation. Theegarten et al (2008) showed that the severity of human chronic obstructive pulmonary disease can be associated exposure to *Chlamydia psittaci* antigens. In a study of lung tissue from RAO affected horses, 60% of were found to have antigens of either C.psittaci or *Chlamydia abortus* by PCR analysis as compared to 45% of healthy horses. RAO affected horses also had a more intense inflammatory reaction to the organism than healthy horses. [123] Obesity has been associated with an increased prevalence of asthma in human females. [124] Airway inflammation was however not associated with body fat. This association has not been investigated in horses with RAO.

1.5 Clinical signs

Horses susceptible to RAO demonstrate minimal signs when in remission. When exposed to inhaled allergens these same horses develop the typical clinical signs which include non-productive coughing, serous nasal discharge, labored expiratory effort, exercise intolerance and flaring of the nostrils. Weight loss can be seen in more chronic cases. Contributing to this situation is the inappetance or reduced feed intake due to a markedly increased breathing effort. The breathing pattern of RAO affected horses consists of rapid inspiration and forced prolonged expiration. Hoffman et al (2007) discovered that RAO horses have a loss of biphasic expiratory flow, reduced contribution of the abdomen to ventilation as well as rib cage/ abdominal asynchrony. [125] Hyperplasia of the external oblique abdominal musculature, commonly known as a "heave line" can be seen in chronic cases. These clinical signs are secondary to the inflammatory response that results in bronchospasm, excessive mucus production and airway obstruction. Clinical signs are often associated with changes in management and rapid reversal of signs associated with pasture turnout or environmental modification. Clinical signs often worsen in winter due to the increased time indoors and poor ventilation together with the feeding of hay stored for longer periods collecting dust.

There are proven relationships between environmental conditions, the practice of hay feeding and the onset of clinical signs. The association of the clinical to environmental conditions or hay feeding supports the belief that RAO is a respiratory allergic disease. A clinical scoring system has been developed in order to grade the severity of clinical signs shown. Both nasal flaring and abdominal effort are graded between 1 and 4 with increasing severity and summed for a total clinical score.[126] However clinical scores only correlate well with more objective measures of disease (such as pulmonary function or changes in breathing pattern) when horses are severely

affected. A total score over 5 was found to identify those horses with considerable pulmonary function changes however the system failed to identify those horses with low-grade airway obstruction. Consequently, clinical score is considered an insensitive method for detecting less severe clinical disease.

Airway inflammation is not an exclusive response of horses with RAO. Healthy horses, exposure to aerosolized debris or extreme environmental conditions may also experience low degrees of airway neutrophilia in that absence of notable respiratory compromise. Robinson et al (2006) evaluated the factors involved in subclinical airway inflammation and found an increase neutrophil count in tracheal lavage samples in horses eating hay, especially round bale hay while horses on pasture had the lowest number of neutrophils. Females kept outdoors in winter had increased numbers of inflammatory cells in the lower airways. Older horses had fewer macrophages than young horses. Greater than 70% of the horses evaluated had neutrophil counts from tracheal fluid of greater than 20%. Approximately a fifth of the horses had a mucus score greater than 1. Risk factors for mucus score greater than 1 included horses more than 15 years of age, hay feeding compared to pasture, and being outdoors more than 80% of the time in winter. [127] While healthy horses experienced airway inflammation in the absence of other clinical signs, many of these conditions are known to induce clinical signs of respiratory compromise and more severe airway neutrophilia in RAO affected horses. Based on these findings, the relationship between airway neutrophilia and equine heaves is not clearly defined.

Robinson et al, 2003 evaluated RAO horses when placed in a stall and determined that they showed an increased cough frequency, mucus score and maximum change in pleural pressure compared to healthy horses which only demonstrated a transient cough when first stalled. The coughing frequency in RAO-affected horses correlated with the maximum change in pleural pressures during tidal breathing (ΔPpl_{max}), the mucus score as well as amount of airway inflammation. The coughing frequency was found to be a sensitive and specific indicator of a $\Delta Pplmax > 6cmH_2O$, a mucus score>1.0 and a BALF cytology differential of > 20 % neutrophils. [128]

1.6 Energy demands

Many horses affected with recurrent airway disease are in poorer body condition than those not affected by the disease. This was confirmed by Mazan and coworkers, where RAO-affected horses had a significantly lower body condition score when compared to healthy horses. By evaluating energy utilization it was shown that RAO-affected horses demonstrated an increased energy demand along with increased oxygen consumption. The amount of oxygen consumption was strongly correlated with indexes of airway obstruction. By administering a bronchodilator to the RAO affected horses the authors were able to show decreased oxygen consumption. The work of breathing is suspected to increase energy demand by the body and result in a lower body condition score.[129]

1.7 Diagnostic methods

1.7.1 Physical examination

A thorough physical examination is required in order to localize the problem to the respiratory system, identify and determine the extent of the characteristic findings, and rule out other potential diseases as well as complicating factors. Clinically affected RAO horses often have abnormal lung sounds such as crackles and wheezes on auscultation and an expanded lung field. Expiratory wheezes are typically detected throughout the auscultation area while crackles are more noticeable at the lung periphery. These abnormal sounds are due to the forceful movement of air through a narrowed airway as well as due to the accumulation of inflammatory debris in the small airways. Percussion of the thoracic wall has been shown to be useful in determining the location of the lung field and in diagnosing a caudal shift of the caudal lung border in horses suffering from RAO. [130] In horses with severe disease the physical examination together with the history may result in a strong suspicion of the presence of RAO while is lower-grade disease or those in remission it will be vital to pursue further diagnostics. Couetil et al. showed that a clinical examination had a low to moderate sensitivity and predictive values for the diagnosis of RAO (Range: 67 to 80%). [131]

1.7.2 Laboratory findings

Analysis of blood samples reveals minimal changes with the majority of RAO affected horses. Some horses however may show changes consistent with endogenous cortisol release. Derksen and coworkers demonstrated that the peripheral blood white cell counts and immunoglobulin/ albumin ratios in RAO affected horses were unaffected by barn exposure and return to pasture. [17] However, a complete blood count and serum chemistry aids in ruling out other diseases affecting the pulmonary system.

Horses with a severe episode of airway obstruction typically show abnormalities on a blood gas analysis. Poor ventilation of the alveoli with no alteration in blood perfusion leads to inefficient gas exchange resulting in hypoxemia with normocapnia. [132] This is compounded by airway inflammation, bronchoconstriction and mucus accumulation. Jean et al (2004) found that horses with an acute episode of airway obstruction demonstrated a metabolic alkalosis with hyperchloremia compared to healthy horses. [133]

1.7.3 Diagnostic imaging

a) Radiology

Obtaining thoracic radiographs may be beneficial during an initial evaluation of RAO affected horse for the purpose of ruling out other respiratory diseases present with clinical similarities to equine heaves. The majority of heavey horses have no abnormal findings on thoracic radiographs with changes often only evident in advanced cases. Clinically affected RAO horses may

demonstrate an interstitial pattern diffusely throughout the lungs with some small airway thickening due to airway remodeling. Peribronchiolar cuffing or other infiltrates can also be seen. Loss of thoracic detail and the degree of opacification of the lung field is considered an indication of the severity of the disease. Hyperinflation of the lung may be present causing flattening or concavity of the diaphragm and a more lucent lung field. Late in the disease it may be possible to see bronchiectasis.[134]

b) Ultrasonography

Ultrasonography can be useful to evaluate the pleural surface and is shown to be reliable in determining the location of the caudal lung borders in horses with RAO. [130] However due to the limitations in penetration this modality can only detect surface pulmonary pathology and is not a sensitive method for evaluating the lung of RAO affected horses. Like radiology, this modality is most useful for ruling out other respiratory conditions or complications.

c) Scintiography

This imaging modality is not commonly used clinically for diagnostic purposes as more convenient and less time consuming methods exist. Scintiography has however been used to evaluate lung perfusion and ventilation; to measure alveolar clearance; determine inflammatory cell involvement in lung disease; assess mucociliary clearance and study the deposition of aerosol medications in the lung. [135]

1.7.4 Endoscopy

Evaluation of the airways via endoscopy allows visualization of the degree of mucus accumulation as well as fibrous thickening of the tracheal bifurcation. Clinical, endoscopic and cytological assessment such as mucus accumulation and airway neutrophilia of lower airway inflammation were shown not to correlate with the thickness of the tracheal septum. Older horses ($\geq$ 10 years) septum scores were significantly higher than younger horses but did not differ between clinically normal and RAO-affected horses both in exacerbation and in remission. [136] A scoring system was devised to assess mucus accumulation in the trachea of horses[137] which was found to correlate well with neutrophilic airway inflammation and to be a reproducible measure of the volume of mucus in the trachea. Endoscopic scoring of mucus accumulation is considered to be a reliable clinical and research tool however the apparent viscosity, localization and color scores should be interpreted with caution.

1.7.5 Exhaled breath condensate

Exhaled breath condensate sample collection from horses is a non-invasive procedure that can be relatively easily performed. This diagnostic tool however is primarily used for research purposes. Deaton et. al (2006) studied exhaled breath condensate by measuring the concentration of hydrogen peroxide and ascorbic acid and correlating this with the extent of inflammation present in the lower airways. Hydrogen peroxide was found to be significantly higher in RAO-affected

horses with marked airway inflammation. The concentration of hydrogen peroxide in the exhaled breath condensate was found to be inversely related to the concentration of ascorbic acid (anti-oxidant) and positively correlated to the BALF neutrophil count and tracheal wash inflammation score. The authors therefore believed that by measuring the level of hydrogen peroxide in exhaled breath condensate it may be used as an indicator of the severity of neutrophilic airway inflammation. [60] However, exhaled hydrogen peroxide has been shown not to be affected by environmental management changes. [40]

1.7.6 Lung biopsy

Collection of a lung biopsy by means of a percutaneous biopsy needle (14 gauge 15cm Tru-cut biopsy needle) or pulmonary wedge resection under thoracoscopic guidance has been described. [138] Both techniques provided good specimens for histological evaluation of the lung and are considered minimally invasive. The sample obtained may be useful on a histological level to further determine the extent and severity of the pathology and to aid determination of the prognosis in the affected horse. Lugo et. al (2006) demonstrated that there was no significant difference between lung regions or groups of horses when evaluated via lung biopsy. There was a lack of regional differences in airway structure indicating that biopsy samples can be used for the diagnosis as well as investigation of diffusely distributed diseases.[50] Complications include epistaxis, pulmonary hemorrhage, tachypnea, respiratory distress or less commonly pneumothorax, collapse or death.

1.7.7 Bronchoalveolar lavage

Bronchoalveolar lavage is a method used to retrieve respiratory secretions that line the small airways and alveoli and forms an important part of the diagnostic work-up of a horse suspected to be affected with RAO. This procedure is performed with a flexible cuffed nasobronchial tube passed blindly into the trachea and advanced into the airways until the tube becomes wedged (mid-bronchi). The cuff of the tube is then inflated to make to make a seal and minimize infused fluid from back-flushing into the larger airways. Sterile saline (300mls) is then flushed and retrieved through the tubing and recovered fluid is analyzed. The procedure can be performed under endoscopic guidance. Samples collected are considered to be representative of the entire lung in horses that are experiencing generalized airway disease, like RAO. [139] Cytological assessment of these samples assists in determining the extent of inflammation which is based on the presence and distribution of immune cells. The procedure is considered safe and sensitive at detecting inflammation. Characteristically horses with RAO have a neutrophilic leukocytosis with the neutrophil count up to 4-fold the number expected in a normal horse. Most clinically normal horses have a neutrophil percentage of the total cell count of less than 10% whereas horses affected with RAO are often found to have greater than 25% neutrophils in the BALF sample. An advantage of this procedure over a tracheal wash is that the BALF cytology correlates well with the clinical signs and pathophysiologic processes of RAO. [17] The cytological composition of fluid obtained by tracheal wash tends to be more variable, although neutrophilia

is still a common observation. [140] Studies consistently find a marked increase in the percentage of neutrophils in BALF collected from RAO affected horses with the percentage of neutrophils increasing with the severity of the condition. [141]

Analysis in dogs showed that several lavages performed at 5- to 7-week intervals were safe and could be used reliably for evaluation of therapeutic efficacy .[142] The effect of a bronchoalveolar lavage procedure on pulmonary function has been evaluated. The procedure induced a significant decrease in pulmonary resistance for up to 6 hours which was thought to be as a result of mucus clearance from the larger airways. A significant increase in lung elastance was also observed. [143]

Various antitussive agents have been evaluated to reduce undesirable coughing during the BALF procedure. Intratracheal administration of lidocaine (0.66%) prior to the procedure resulted in the most reduction in coughing frequency. Intravenous administration of butorphanol or intratracheal administration of 0.33% lidocaine resulted in a nonsignificant reduction in coughing frequency. Other treatments failed to significantly suppress coughing frequency and intensity. Glycopyrrolate caused obvious adverse clinical effects such as transient heart murmurs, reduced gastrointestinal sounds and mydriasis. Treatments did not influence the volume of BALF collected or the composition of the fluid. [144]

1.8 Bronchoalveolar lavage fluid cytology

The cytological evaluation of the BALF is one of the most important diagnostic tools in the investigation of RAO. Pickles and coworkers determined that even low BALF recovery volumes remained diagnostic. Once a BALF sample has been obtained it is important to process the sample appropriately in order to gain the most information from the cytological assessment. All collected aliquots can be considered representative of the cytological composition of the lavaged lung segment. [145] In order to preserve cell morphology it is necessary to keep the sample on ice and process the sample within a short period of time. Cell viability was shown by Pickles et al. to be influenced by time period prior to fixing as well as the storage temperature and a delay in processing does predispose to bacterial growth. [146] The fluid obtained is often very dilute and so direct smears are often unrewarding. Centrifugation of the sample and making a smear of the cell pellet, or alternately using a commercial cytospin machine is recommended. Cytological identification is considered more challenging in a smear as compared to cytospin preparation but smear preparation does permit reliable in the diagnosis of RAO. [145,146]

The cellular composition in horses affected with RAO is characterized by neutrophilic leukocytosis. Airway neutrophilia is dose dependent depending on the level of airway allergen exposure. [38,39,147] There is no significant difference between BALF from healthy horses and RAO affected horses in remission in total nucleated cell count or relative percentages of small lymphocytes, large mononuclear cells, eosinophils, or mast cells. The mean relative neutrophil count of BALF from clinically affected RAO horses is significantly greater than healthy horses. However, the difference between healthy and RAO affected horses is not considered sufficient to

be of diagnostic significance. [148] Therefore the percentage of neutrophils in BALF is highly sensitive (100%) and moderately specific (64%) for the diagnosis of RAO. [131] This implies that cytological evaluation allows for early detection of inflammatory conditions of the respiratory system but is not specific for RAO.

Equine RAO shares a number of similarities with human asthma but eosinophils are less commonly associated with the heaves than asthma. Young horses affected with inflammatory airway disease demonstrate airway eosinophilia along with airway hyperresponsiveness similar to children with asthma and some authors speculate that this condition in horses may represent early events in the progression of RAO. [149] Mast cells are also rarely seen in BALF collected from RAO-affected horses, however this may be a false negative finding as it has been shown that equine mast cells are not well visualized with most commonly used staining techniques. Special staining can improve identification of these cells but is rarely performed due to the interference with visualization of other cellular components. In human asthma mast cell degranulation plays a pivotal role in the response to allergen. In both normal and RAO affected horses mast cell degranulation and release of tryptase also occur in response to challenge.[122]

1.9 Pulmonary function testing

Pulmonary function testing is an extremely useful method of objectively evaluating RAO horses in response to challenge. Challenge studies in RAO-affected horses have shown repeatable airway obstruction and marked differences between healthy horses and those affected with RAO when evaluated using this procedure. The severity of disease as well as response to therapeutics can be also objectively determined. Despite its advantages however, this method is fairly costly, time consuming and requires technical expertise which limits the use of the procedure to mostly research. Age, sex and usage of the horse effect pulmonary function and need to be considered when establishing the pulmonary functional status in horses with clinical RAO. [150] The procedure involves the use of an esophageal balloon placed on the end of a 5mm tube catheter which is passed into distal third of the horse's esophagus. The tubing is a closed system and exits through a sealed portion in an airtight mask attached to the horse's muzzle. A transducer is placed in the mask and measures air velocity as the horse inhales and exhales. This information is converted to electronic signals by two separate transducers (one for pressure and one for flow). The transducer attached to the esophageal balloon measures pressure changes within the esophagus which estimates the variation in intrapleural pressure required to maintain airflow in the presence of obstruction of the lower airways. The flow transducer measures air flow through the nose and these measurements are then used to calculate additional aspects of pulmonary function such as dynamic compliance, tidal volume, resistance and elasticity. The maximal change in pleural pressure difference between inspiration and expiration during tidal breathing (ΔPpl_{max}) can be derived through these methods and is frequently used as an indicator of pulmonary function and the work of breathing. Normal horses typically have a ΔPpl_{max} of below 10 cmH_2O while horses experiencing an acute episode of airway obstruction often have ΔPpl_{max} in excess of 15 cmH_2O and this has been used to evaluate response to treatment.

Pulmonary mechanics are significantly different in the clinically affected RAO horse compared to healthy horses. However, few differences have been shown between horses with inflammatory airway disease (IAD), RAO affected horses in remission and healthy horses. RAO affected horses demonstrate significant changes in clinical score, respiratory rate, peak tidal inspiratory and expiratory pressures, dynamic compliance and pulmonary resistance when exposed to a challenge environment. [151] Increasing airway obstruction in response to natural challenge such as housing susceptible horses in a stall is reflected by increasing pleural pressures and pulmonary resistance with a decreasing dynamic compliance [152] and PaO_2. [16,153,154] RAO affected horses have demonstrated a significantly higher expiratory to inspiratory ratio. Horses with recurrent airway disease have also been shown to demonstrate increased airway hyperresponsiveness during acute exacerbations of airway obstruction. However there is no correlation between this airway reactivity and changes in pulmonary resistance and dynamic compliance. Environmental modification or treatment with atropine cause marked improvements in pulmonary function tests but the response to the administration of atropine in RAO affected horses underestimates the actual improvement in respiratory tract function that occurred when horses where maintained on pasture .[155] Pulmonary lung function testing is thought to have a low sensitivity and predictive value [131] when compared to more advanced techniques such as forced expiration techniques.

Herholz and coworkers assessed the ability to evaluate pulmonary function via volumetric capnogram findings. This method was not found to have superior sensitivity or specificity as compared to the conventional pulmonary lung function testing when differentiating between clinical degrees of RAO in horses. [150]

Forced expiration techniques

The use of forced expiration techniques in RAO affected horses is described. Originally the procedure was performed in anesthetized horses but is more recently described in standing sedated horses. A nasotracheal tube as well as esophageal balloon catheter is placed. The nasotracheal tube is then connected to three way value with ports leading to a mechanical ventilator, or vacuum reservoir. The head and neck are kept extended throughout the procedure. Alternating between mechanical ventilation and the vacuum allow for evaluation of pulmonary parameters. [156] The majority of variables for forced expiration in horses affected with RAO or IAD differed significantly from healthy horses. This method has a high sensitivity, specificity and predictive value (79 to 100%) for the diagnosis of RAO. Horses in the early stages of the disease are therefore thought to be better detected by this method. [131]

Impulse oscillometry

Due to the poor sensitivity of conventional pulmonary lung function testing a new method known as impulse oscillometry offers an alternative to pulmonary lung function testing. An airtight system with minimal deadspace is vital to impulse oscillometry testing. Measurements are repeatable and not impacted by the size of the horse. As in conventional pulmonary lung

function testing the impulse oscillometry parameters in RAO affected horses in crisis differed significantly from values during remission. When this method was compared to conventional pulmonary function testing it was found that impulse oscillometry parameters were significantly more sensitive for testing pulmonary function. This method is quick, sensitive and minimally invasive and may be a promising means for assessing subclinical changes in affected horses. [157,158]

Flow loops

Tidal breathing flow volume loops are generally considered a research modality. Tidal breathing flow loops in RAO horses both in remission and under increasing amounts of airway obstruction are available. Clinically healthy horses typically demonstrate biphasic inspiratory and expiratory patterns with peak inspiratory and peak expiratory flows detected early in their respective phases. Tidal volume was shown not to be affected by RAO in the horses evaluated while respiratory frequency was increased due to a reduced inspiratory time. RAO affected horses did not demonstrate a biphasic pattern and the peak inspiratory flow was only observed late in inspiration and peak expiratory flow early in expiration. As airway obstruction increased, tidal breathing flow volumes demonstrated a characteristic appearance with a peak expiratory flow early in expiration followed by a low flow rate. [159] Factors such as the use of the horse do affect the ability of tidal breathing flow volume loop indices to diagnose different degrees of RAO. [160]

1.10 Current treatment options

1.10.1 Environmental modification

Any exposure to inciting airborne allergens in RAO susceptible horses can result in a rapid cascade of events leading to the acute onset of a respiratory crisis. Important factors include the quantity of antigenic material inhaled as well as the sensitivity of the individual horse to specific antigens. RAO affected horses show a greater response to allergens along with an increased degranulation of neutrophils compared with clinically normal horses. This is thought to play a central role in the pathogenesis of RAO making prevention a critical element in treatment of the disease .[149] Although complete allergen avoidance is ideal, this is generally not achievable. A more realistic goal is to limit exposure as much as possible through environmental modification.

A low dust environment is the treatment of choice as this limits the exposure to airborne dusts. Harmful airborne particles incriminated in RAO have been shown to have a diameter of less than 5 micrometers. Larger particles are typically trapped in the mucus of the upper airways. Ideally RAO affected horses should be housed on pasture as this environment is the best in terms of ventilation and elimination of exposure to airborne allergens. Jackson et al demonstrated that significant improvement in lung function was seen within 3 days of a change to pasture and this continued to day 7 with airway function being best by day 30 [161,162] emphasizing the importance of this intervention in the treatment of RAO. Others have shown that neutrophil counts in BALF may take as long as 2 weeks on pasture to decline. While pasture is the best environment for

most RAO affected horses, this option is not always available due to space limitations or competitive commitments. Minimizing allergen exposure in the indoor environment necessitates evaluation of factors such as bedding, hay, ventilation and adjacent stalls [163] in order to improve the horse's environment to the extent that will result in clinical improvement.

Hay is an important source of dust and mold. Ideally hay should be eliminated from an RAO affected horses environment. Alternatives include the use of pelleted or cubed hay sources or making use of hay silage. However exposure to clostridial toxins is a concern when hay silage is fed. Soaking hay prior to feeding is shown to reduce respirable dust concentrations [163], but this can reduce the nutritional content of the hay. Complete diets in the form of a commercial pelleted feed are available and nutritionally formulated to allow the RAO affected horse to be fed this diet without access to forage. These diets are low in dust and eliminate exposure to hay feeding but shorten the time over which horses consume their food.

Bedding is another important aspect of environmental modification. The type of bedding material as well as its maintenance is important. Avoiding moisture accumulation is paramount in minimizing mold growth. The rates of release of fungal and mold spores from hay and bedding, as well as the rate of clearance from the stall have been evaluated. [164] The amount of respirable particles in the stall increases significantly if there is increased fungal contamination of the bedding. [23] The hay is the most usual source of contamination of the bedding. When the different types of bedding were assessed, Webster and coworkers found only a small difference in respirable dust particles between straw, wood shavings and paper bedding in stalls where bedding was properly selected and stored.[164] Stall cleaning which caused a disruption of the bedding increased the concentration of respirable dust in the horse's immediate environment by 3-6 times. Other studies have shown that the concentration of stall dust in barns using straw is higher and more variable than for wood shavings. [165] Straw also has a higher content of molds and endotoxins than good quality wood shavings.[21] Alternatives include paper, cardboard, peat moss and non-biological agents. Price, disposal and the risk of intestinal obstructions are also important considerations.

Ventilation in the horses' environment has been found to be the most important factor in clearance of stall dust. If the rates of release of stall dust from sources such as bedding and hay are low then ventilation rates over 4 air changes/hr are satisfactory. [164]

Miskovic et.al (2007) evaluated the long term maintenance of RAO susceptible horses in a low dust environment without additional medical management. Clinical scores remained higher in RAO affected horses 2-3 years following a diagnosis when compared to healthy horses. Conventional lung function tests did not differ between the RAO susceptible horses and healthy horses. Forced expiratory flow was shown to be reduced between 75-95% of exhaled vital capacity in the RAO susceptible horses when compared to the healthy horses illustrating that there is persistence of airway obstruction. BALF cytology did not show any differences in total neutrophil count or differential counts between the RAO affected horses and healthy horses at

any stage. The authors therefore concluded from the study that the peripheral airway obstruction that was detected in RAO affected horses maintained in a low dust environment was likely due to irreversible airway remodeling that was not associated with evidence of airway inflammation [166].

1.10.2 Drug administration

The addition of periodic bronchodilator and anti-inflammatory corticosteroid therapy is often required in acute episodes of airway obstruction. Drug administration may induce a quicker onset of remission and thereby avoid the potentially detrimental effects of severe inflammatory reaction. Such drugs are effective in the majority of cases but unwanted adverse effects should be considered prior to implementation into a treatment program.

1.10.2.1 Anti-inflammatory medications

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex. Natural corticosteroids in the body are typically classified into glucocorticoids (GCs) (e.g. cortisol) and mineralocorticoids (eg. aldosterone). A variety of synthetic compounds have been created for therapeutic use which have similar actions to the natural hormones in the body. By far the most important use of this class of medication is an anti-inflammatory agent that is used both in human and veterinary medicine. Glucocorticoids act by binding to cellular GC receptors which are found on the surface of almost every cell. Bronchial epithelium and vascular endothelial cells have a large amounts number of GC receptors (Barnes 1996).The anti-inflammatory properties of GC are thought to be mediated by suppression of inflammatory gene expression via inhibition of transcription factors such as NF-κB and AP-1.[167] It is not known which specific genes are responsible for the anti-inflammatory activity of glucocorticoids. There is mediation by stimulation of transcription of genes with anti-inflammatory actions as well as inhibition of transcription of genes with inflammatory activity. Glucocorticoids also inhibit the expression of a number of cytokines including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, IL-16, granulocyte/monocyte colony stimulating factor (GM-CSF), tumor necrosis factor-a, matrix metalloproteinase 9, and the chemokines eotaxin, macrophage inflammatory protein and monocyte chemoattractant protein 1. [168] Inhibition of the downregulation of β2 adrenoreceptors, T cell proliferation and humoral immunity occurs. B cells secrete less IL-2 which diminishes B cell clone expansion and antibody synthesis. Decreased IL-2 results in less activation of T cell lymphocytes. GC also have profound negative effect on various macrophage functions. Inhibition of IL-1 production and decreased expression of Fc receptors results in decreased phagocytosis of opsonised cells. Another theory for the anti-inflammatory mechanism of GC was thought to be the synthesis of lipocortin-1. Lipocortin-1 suppresses phospholipase A2 thereby blocking eicosanoid production and inhibition of various leukocyte functions such as migration, chemotaxis, phagocytosis and respiratory burst.

The use of GC in RAO affected horses target inflammation in the lower airways and is the most effective of the currently available treatments [169]. In horses however, adverse effects such as a poorly documented association with laminitis; adrenal suppression; increased susceptibility to

infection and gastrointestinal ulceration are of concern. Sodium retention can also occur in which can result in protrusion of the infraorbital fat pads. GC can be administered to RAO affected horses by inhalation or systemically. Daily doses are given for 1-2 weeks followed by a gradual reduction prior to discontinuation. This protocol is dependent on the severity and duration of the disease.

Inhaled GCs in humans are shown to decrease eosinophils, and down regulate Th2 cytokines but increase neutrophils in airway mucosa. [170] Glucocorticoids fail to inhibit IL-8 mRNA expression and this may allow persistence of airway neutrophilia despite treatment with glucocorticoids. [170]

Systemic glucocorticoids

a) Dexamethasone

Dexamethasone is a synthetic GC with minimal mineralocorticoid activity. It has approximately 25 times the anti-inflammatory potency of naturally occurring cortisol. Dexamethasone results in a rapid relief of airway obstruction and can be administered via injection, inhalation or oral route depending on the product. The dose range is 0.05mg/kg to 0.1mg/kg [162,171] orally or 0.04mg/kg daily intravenously (IV). [110] The same dose (0.04mg/kg) given intramuscularly (IM) is effective but there is a risk of severe infection. Adrenal suppression in RAO affected horses can result from the administration of 25mg of dexamethasone intramuscularly on alternate days for 4-6 treatments. [169,171] Adrenal suppression typically occurs within 2 days of treatment but resolves quickly following discontinuation of the medication. [171] Due to its increased potency dexamethasone is potentially associated with more adverse effects than less potent agents.

Dexamethasone (0.1mg/kg, IV, q 24 h) causes significant improvement in lung function of affected horses within 2 hours with a peak effect at 4-6 hours. Following 7 days of treatment pulmonary function testing results are similar to those of the same horse on pasture.[162] Airway neutrophilia is decreased following one week of dexamethasone at a dose of 0.1mg/kg IV once a day. [162] Rebound to pretreatment values is seen following discontinuation of the medication if the horse remains in a challenge environment. [171] The use dexamethasone in RAO-affected horses decreases the cough frequency, mucus score as well as ΔPpl_{max}.[128]

Dexamethasone administered orally (0.164mg/kg prior to feeding) was effective within 6 hours with a peak effect at 24 hours. The duration of effect was approximately 30 hours. Oral administration of a bioequivalent dose of the same solution of dexamethasone as typically administered intravenously is as effective as when administered IV but has a longer duration of action. Fasting prior to administration of oral dexamethasone improves efficacy of treatment. [172] The use of oral prednisolone (1mg/kg) or dexamethasone (0.1mg/kg) intramuscularly when combined with environmental management has similar effects on clinical scores, blood gases and endoscopic scores. Dexamethasone had a slightly more beneficial effect on BALF cytology when compared to prednisolone. [173]

A dietary change from hay to pelleted feed has been compared to a 21 day decreasing dosage regimen of oral dexamethasone (0.165 mg/kg PO q24h for a week then 0.083 mg/kg PO q24h for a week followed by 0.04 mg/kg PO q24h for a week). Both treatments reduce airway neutrophilia and improve respiratory parameters. The addition of dexamethasone was associated with fewer treatment failures. [174] Both treatments reduce the expression of IL-8 and CXCL2 in the airway epithelium, but fail to alter the expression of IL-1 R2 and TLR-4.

b) Prednisone and prednisolone

Based on the efficacy of prednisone for the treatment of human asthma, the corticosteroid was investigated as a treatment for RAO in horses. Studies failed to show significant benefit of prednisone tablets in improvement of clinical signs, pulmonary function or BALF cytology in RAO affected horses. [175,176] Oral absorption of the drug was determined to be extremely poor with little to no conversion of prednisone to it active form, prednisolone.[162] Some clinical benefit was associated with the use of prednisone tablets however it was significantly inferior to the use of dexamethasone and took a much longer time period to take effect.

The use of the corticosteroid, prednisolone orally (1-2 mg/kg every 24 hours) is rapidly absorbed, detectable levels in serum within 15 min of administration, and reaches a peak concentration within 45 min. [177] Prednisolone induces a rapid reduction in airway inflammation with a slower improvement in airway function. [175] Bacterial pneumonia is reported as a rare complication of long term prednisolone (1mg/kg q48hrs) [178] treatment. This may not be exclusive to prednisolone but rather a result of the overall immune suppression of the class of GCs.

c) Triamcinolone

Triamcinolone (0.09mg/kg) IM improves respiratory function of clinically affected RAO horses maintained in unfavorable environments for up to 3 weeks.[169] However, adrenal function is altered for up to six weeks. It is recommended that a second dose should not be administered within 6 weeks after the initial injection to avoid complications.

d) Isoflupredone acetate

Isoflupredone acetate is a potent corticosteroid with both glucocorticoid and mineralocorticoid properties. Isoflupredone acetate (0.03mg/kg, IM, q 24 h) is as effective as dexamethasone in the treatment of RAO affected horses but is associated with hypokalaemia. Adrenal suppression is longer with isoflupredone than with dexamethasone. [110]

Inhaled glucocorticoids

Inhaled medications may be used to avoid the risks associated with long-term administration of systemic corticosteroids. This method of administration is the first line of treatment in human asthma and is considered to be safe. [179] When evaluated in horses inhaled GCs have been found

to be safe and effective. [86,161] Oral candidiasis is considered a potential side effect of inhaled GCs in humans however this has not been reported in horses. Several drugs have been administered via metered dose inhalers with the use of a mask. Advantages include a reduced systemic absorption and shorter residual time which are important factors in competition horses. Disadvantages include the expense of treatments and time required for treatment.

e) Beclomethasone diproprionate

Inhaled beclomethasone is shown by to be effective in RAO affected horses by reducing clinical signs of airway obstruction as well as improving pulmonary function testing responses.[180] The magnitude of response to inhaled beclomethasone is considered to be inferior to parenterally administered dexamethasone. Inhaled beclomethasone improves respiratory effort after 2 days of treatment and BALF neutrophil counts following 10 days of treatment. A rebound to pretreatment values occurs when treatment ceases if the horse remains in a challenge environment. [171] Low dose inhaled beclomethasone (500µg q12hr) failed to have an effect on the BALF cytology, NF-κB or AP-1 activity in the airways of RAO affected horses. [181] There was no immediate effect following administration of incremental doses of inhaled beclomethasone diproprionate to RAO affected horses. However, within the first 24 hours of receiving the medication there was a decreased amount of airway obstruction coupled with a reduction in ΔPpl_{max}.[180] Beclomethasone administered via inhalation also decreased serum cortisol levels, indicating that that systemic absorption occurred.[171] Administration of inhaled beclomethasone resulted in adrenal gland suppression within 2 days of treatment in a dose dependent manner. The suppression rapidly resolved after discontinuation of treatment (2-4 days).

f) Fluticasone propionate

Inhaled fluticasone propionate improves the clinical signs, pulmonary mechanics and BALF neutrophil count when administered to clinically affected RAO horses. [86] This is associated with a reduction in IL-4 expression and an increase in the IFNγ/IL-4 ratio in the airways. Treatment with inhaled fluticasone in severe RAO results in a more rapid improvement in pulmonary function. Early treatment with inhaled fluticasone may accelerate the recovery of horses with severe RAO.[182]

1.10.2.2 Bronchodilators

Bronchodilators form an integral part of the treatment protocol in human asthma. The use of this class of medications is also useful in the management of RAO affected horses, especially during the acute phase of airway obstruction. Bronchodilators alone are considered to be contraindicated in horses that remain in the same environment as it is thought that this allows an increased number of allergens to reach the lower airways. Bronchodilators target airway smooth muscle and some medications possess additional anti-inflammatory properties. Anticholinergic agents, β2 adrenergic agonists and methylxanthines are most commonly used.

The distribution of aerosolized medications was therefore assessed by the use of scintographic methods. The distribution of aerosolized radiolabelled markers was determined to be non-uniform when administered to acutely affected RAO horses. Specifically, poor penetration of peripheral lung fields and an excess deposition in the large airways was observed. Administration of a bronchodilator prior to administering a radioactively labeled aerosol agent caused improvement in pulmonary distribution. This is important in the administration of aerosolized corticosteroid medications where a bronchodilator prior to the corticosteroid results in more even distribution of the treatment.[135]

The metered dose inhalers originally developed for humans with asthma are easy to use and result in rapid inhalation of medications in horses. [183,184]

a) Pirbuterol acetate

When given to RAO horses during an acute episode of airway obstruction this β-2 adrenergic agonist induces a marked improvement in pulmonary resistance, dynamic compliance, minute ventilation and ΔPplmax for up to one hour following treatment.[183] Signs such as sweating, trembling and excitement are only seen at high doses. An immediate response is seen with no adverse effects at the recommended dose, making it a safe and effective treatment in horses with RAO.

b) Albuterol

Albuterol given via aerosol is safe and effective as a bronchodilator in horses. This drug has a rapid onset of action (5min) and the effects last up to 3 hours. No adverse effects are associated with commonly used dosages. [185] The use of albuterol in RAO affected horses can significantly decrease resting energy expenditure by decreasing the work of breathing.[186]

c) Trimetoquinol

Trimetoquinol is a β-2 adrenergic agonist that is popular in the human market for treatment of asthma. Intravenous administration in RAO affected horses resulted in moderate bronchodilation and a marked elevation in heart rate due to the potent cardiac stimulatory effects of this medication. The onset of action was very rapid and the duration of effect was short. [187] When evaluated via an aerosol route of administration trimetoquinol was shown to have a dose dependent bronchodilatory effect with no change in heart rate or other adverse effects. Given via aerosol this medication is considered to be safe and effective as a bronchodilator in horses. [188] Oral administration is ineffective in horses.

d) Theophylline

Theophylline is from the methylxanthine class of drugs. This medication is thought to possess corticosteroid sparing effects in the treatment of asthma in humans allowing a reduction in the dose of corticosteroids when used in combination. In horses affected with RAO this effect is not

appreciated. Theophylline (5mg/kg PO q12h) failed to improve lung function in RAO affected horses. [189]

e) Salmeterol

The use of this drug in horses affected with RAO resulted in a reduction in pulmonary resistance and change in pleural pressures that lasted for approximately 6 hours. [190]

f) Clenbuterol HCl

Clenbuterol is a β-2 agonist that has been used both in human and veterinary medicine as a bronchodilator. Clenbuterol can be administered orally or intravenously. Intravenous administration in horses can result in adverse effects and therefore the drugs should to be administered very slowly. Oral dosages are 0.08 microgram/kg up to 3.2microgram/kg. Side effects include trembling, sweating and nervousness at high doses.[191] Up to a quarter of RAO affected horses don't respond to this treatment. [191]

Clenbuterol has bronchodilatory, anti-inflammatory, growth promotional and improved mucociliary clearance effects. Administration of Clenbuterol orally results in significant improvement in clinical parameters of RAO affected horses that respond to this therapy. If used as the only treatment in clinically affected RAO horses clinical signs relapse after drug withdrawal. [191] Clenbuterol demonstrates anti-inflammatory properties in addition to bronchodilatory effects with a significant improvement in lung function, total cell count, reduced IL-1β expression and BALF neutrophil counts following its use. The anti-inflammatory effect of clenbuterol is considered to be a valuable additive effect in the treatment of horses with RAO. [36,192] Clenbuterol suppresses lipopolysaccharide induced IL-10 mRNA expression in peripheral white blood cells and TNF-α production by alveolar macrophages. [90] Clenbuterol also increase mucociliary clearance but only for short duration.[193] Clenbuterol administration is prohibited by many racing authorities due to its effects of growth promotion and stimulatory properties. A sensitive method of detecting clenbuterol in the urine of horses up to 36 hours post treatment exists. [194] This must therefore be considered and adhered to in horses competing in racing events.

g) Phosphodiesterase inhibitors

Phosphodiesterase inhibitors have been shown to be beneficial in a number of inflammatory conditions, to inhibit TNF-α and to specifically inhibit equine neutrophil function *in vitro*. The phosphodiesterase-4 enzyme inhibitors have anti-inflammatory properties that were originally thought be beneficial in horses with RAO. A number of phosphodiesterase-4 inhibitors have been evaluated in RAO affected horses, including L-826,141[195], rolipram, pentoxifylline[196] and zaprinast. [197] L-826,141 inhibits up to 90% of TNFα and LTB_4 in fresh blood after endotoxin induction *ex vivo*. However L-826,141 did not improve lung function or have a significant effect on BALF cytology in RAO affected horses. Rolipam also has little effect on lung function and neutrophil accumulation in RAO affected horses. Zaprinast does not result in significant improvement in clinically affected RAO horses. [197] Pentoxifylline is poorly absorbed in horses

with a noticeable variation in serum concentrations over time and among horses. Pentoxifylline reduces pulmonary resistance in RAO affected horses but has no effect on BALF cytology. Administration at high doses improves respiratory function in horses maintained in unfavorable environments but it is uncertain whether this effect is due to anti-inflammatory or bronchodilatory effects. [196]

1.10.2.3 Other treatment options

a) Xylazine

Xylazine is an alpha-2 adrenergic receptor agonist that is commonly used for sedation in horses. Alpha-2 adrenergic receptor stimulation causes presynaptic inhibition of cholinergic nerves innervating the distal portions of the bronchi of horses. [198] No effects on pulmonary resistance are seen in healthy horses or RAO horses in remission following the administration of xylazine.[199] Xylazine (0.5mg/kg) intravenously results in a significant decrease in pulmonary resistance and an increase in dynamic compliance in clinically affected RAO horses. No effect on arterial oxygen or carbon dioxide partial pressures occurs.

b) Atropine

Atropine is an anticholinergic drug that acts as a competitive antagonist for the muscarinic acetyl choline receptor. Broadstone and coworkers showed that atropine failed to alter pulmonary resistance, dynamic compliance or airway responsiveness in normal healthy ponies or RAO horses in remission. During an acute episode of airway obstruction atropine administration did not alter dynamic but reduced pulmonary resistance. [76] The disadvantage of the use of atropine in horses is the adverse effect of the drug on gastrointestinal motility which can lead to colic.

c) Furosemide

Furosemide (1.0 mg/kg) is a loop diuretic that has been used both intravenously and via aerosol in horses during the acute period of airway obstruction. A significant reduction in pulmonary resistance and increase in dynamic compliance regardless of route of administration is reported. No changes in either arterial oxygen or carbon dioxide partial pressures or respiratory frequency were seen. [200] The drug demonstrated no effect on normal healthy horses or RAO affected horses in remission. When administered intravenously the beneficial effects began 15 min after administration and lasted up to 5 hours. The use of the non-steroidal anti-inflammatory agent, flunixin meglumine (1.1mg/kg q8h) abolished the effect of furosemide on airway obstruction but did not prevent diuresis. This suggests that the effect of furosemide is mediated through prostanoids and is not due to the diuretic effect.[201]

d) Lidocaine

Lidocaine is a local anesthetic. While aerosolized lidocaine has been used in humans to decrease bronchospasm, this drug has not been evaluated as a treatment for RAO.

e) Biological products

Van den Hoven and coworkers found that an extract of thyme and primula resulted in an improvement in dynamic compliance, pleural pressure changes and pulmonary resistance after one month of oral administration. Clinical signs and arterial partial pressure of oxygen were unchanged by this treatment. [202] Another product containing garlic, white horehound, boneset, aniseed, fennel, licorice, thyme and hyssop did not improve airway mechanics in symptomatic RAO horses when fed for 3 weeks, although there was a reported trend towards a decreased respiratory rate and increased proportion of macrophages in tracheal samples. [203] An oral preparation consisting of extracts from yellow gentian, garden sorrel, verbena and common elder was shown by Anour and coworkers to reduce sensitivity of the airway to histamine in some of the horses evaluated and a significant decrease in ΔPpl_{max} in all horses evaluated. No change in clinical signs, mucociliary activity or BALF cytology occurred. [204]

f) Oxygen

Oxygen therapy may be considered in acutely affected RAO horses in which dyspnea is evident. The use of nasal oxygen is generally well tolerated. Wilson et.al demonstrated that RAO horses that the partial pressure of oxygen increased significantly in all RAO horses supplemented. However, the partial pressure of oxygen remained lower in RAO affected horses when compared to healthy horses. [205] A large arterial end tidal gradient for carbon dioxide in RAO affected horses was also demonstrated, indicating an increase in alveolar dead space ventilation associated with this therapy.

g) Acupuncture

This treatment modality has received a lot of attention as a treatment approach in human asthma however, a single treatment during an acute period of airway obstruction in horses failed to show any beneficial effect. [15]

h) Nutritional supplementation

Polyunsaturated fatty acids have been found to have an ability to modulate the inflammatory response in horses affected by recurrent airway disease. BALF cells from horses supplemented with dietary polyunsaturated fatty acid supplementation in the form of corn oil demonstrated an increased synthesis of increased PGE_2 in response to endotoxin stimulation as compared to cells from horses supplemented with fish oil. TNF_{α} produced by BALF cells in response to LPS stimulation was higher for both the corn oil and fish oil supplemented horses. Phagocytic activity of the BALF cells remained

unchanged. [206] Sunflower oil and seal blubber oil supplementations has been shown to alter the omega 6: omega 3 fatty acid ratios in plasma and in leukocyte cell walls with little effect on clinical signs or pulmonary function. [207]

i) Saline

Overhydration with saline fluids has been shown not to be effective in improving pulmonary lung mechanics in RAO-affected horses.[133]

1.11 Study Rationale and Findings

Equine RAO is a debilitating and incurable disease of horses. Treatment with GCs such as dexamethasone has been shown to improve respiratory parameters of RAO affected horses. Due to the concern of side effects associated with GC therapy, treatment is often initiated with higher doses, followed by a gradual reduction in dose and frequency until the lowest effective amount and longest effective treatment interval are determined. While this approach is commonly used in the treatment of RAO horses and human asthmatics, many questions are unanswered regarding its effectiveness. It is not known how the response to diminishing doses of dexamethasone compares to environmental modification alone, or if a resolution of clinical signs is associated with a reduction in airway neutrophilia. In addition, the benefit of combining a diminishing dexamethasone dose regime with change in environment has not been explored. The goal of this study was to compare RAO horses' response to one of three treatments: 1. Diminishing doses of dexamethasone alone, 2. Diminishing doses of dexamethasone with a change in environment, and 3. Change in environment alone. Our study has revealed several major findings related to the treatments we evaluated.

1. In the absence of an environmental change, RAO horses receiving GC therapy may appear to be in clinical remission as the dose of dexamethasone is reduced even though they are experiencing significant airway neutrophilia. The airway neutrophilia present in horses receiving dexamethasone every third day was especially severe and statistically elevated from the pretreatment value, when horses demonstrated the most evidence of respiratory compromise. These findings suggest that the clinical response of horses treated with GC alone may be perceived as good, especially by novice owners, while significant airway inflammation (55%) is present. Whether this inflammation results in long term damage to the horse's lungs is not known.

2. This response was present in our horses while they were housed in an environment that was subjectively comparable to the average boarding stall and management conditions of performance horses with limited turnout time on pasture. This observation suggests that change in environment is a mandatory component of treatment if the goal is to decrease airway inflammation in conjunction with controlling signs of clinical disease.

3. The response to change in environment was rapid and comparable to that of horses treated with a combination of diminishing dose dexamethasone with change in environment. This is further evidence of the importance of change in environment.

4. Change in environment in this study was not defined as returning horses to full time pasture turn-out. Instead, horses remained in the same stall as that which was used to induce clinical RAO. However, the stall ventilation was improved by opening the window/door at the back of the stall. In addition, horses were provided accesses to a small dirt 12 x 15 ft individual run, and stall bedding and feed were changed to commercially available products. Our findings provide evidence that the barn environment can be adequately modified to produce significant improvement of the horse's condition, and that this modification is an essential component in the effort to reduce airway inflammation.

The results of this study are directly applicable to situations encountered in practice and provide the equine veterinarian with additional evidence based information that will assist in the decision making process involved with the long-term care of the RAO affected horse. Findings described in this study also have application to human asthma, and may serve to improve treatment of affected human patients as well.

Chapter 2

Comparative efficacy of three common treatments for equine recurrent airway obstruction.

Laura C Lee, BVSc[1]

Virginia A Buechner-Maxwell, DVM Diplomate ACVIM[1]

Undine Christmann, DVM PhD Diplomate ACVIM[1]

Sharon Witonsky, DVM PhD Diplomate ACVIM[1]

Kurt L Zimmerman, DVM Diplomate ACVP[2]

Stephen R Werre, PhD[2]

Departments of Large Animal Clinical Sciences and Biomedical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA[1]

Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA[2]

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Corresponding author: Virginia Buechner-Maxwell, Departments of Large Animal Clinical Sciences and Biomedical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA, 24061-0442; e-mail bmax@vt.edu

Introduction

Equine recurrent airway obstruction (RAO) is a respiratory disease of horses that like human asthma, is associated with exposure to inhaled allergens. The disease typically affects older horses and develops in association with stall confinement. [1,27] RAO is reported to affect up to 50% of the horse population worldwide. [7] Poor performance and potential chronic respiratory or cardiovascular changes are linked to the disease. [8,9]

Clinical signs of RAO develop following exposure of the susceptible horse to inhaled allergens. [21] Typical signs demonstrated by affected horses include coughing, nasal discharge, exercise intolerance and increased breathing effort. Stall dust, endotoxins [30,208], fungal elements and molds[28,31] have been incriminated in the etiology and are typically linked to the conditions in the stall environment [41]and the act of hay feeding. [23] The pathogenesis of the disease is essentially a pulmonary hypersensitivity reaction which results in the characteristic findings of lower airway inflammation, bronchospasm, airway hyperresponsiveness and excessive mucus production. Methods of characterizing the severity of the disease include grading systems for mucus accumulation within the airways [136,137], extent of abdominal push involved in the act of breathing and the degree of nasal flaring shown. These methods attempt to allocate a score to these parameters in order to categorize each horse and compare response to treatment. Clinical assessment however has been shown to lack sensitivity when used to assess improvement based on these findings alone. [126] BALF is easy to collect and its cytological assessment not only forms an important part of the diagnostic evaluation of the RAO affected horse but allows for determination of response to treatment by being an objective measure of airway inflammation. [17,98,128,141,152] Pulmonary lung function testing requires specific equipment and technical expertise but is an important adjunct to BALF cytology in monitoring pulmonary parameters of an RAO affected horse whereby clinical assessment is not adequate in tracking subtle changes. Pulmonary lung function testing determines the pulmonary mechanics and the effect of disease or treatment on the lungs ability to function appropriately.[151,166] Classically RAO-affected horses during an acute crisis demonstrate an increased ΔPpl_{max}, increased pulmonary resistance (RL) and decreased C_{dyn}.

RAO affected horses typically require long term management. Environmental modification remains the mainstay of treatment by minimizing exposure to inciting allergens. [182] GCs are medications that effectively attenuate clinical signs and the airway response when environmental changes cannot be achieved. GC treatment may also speed resolution of disease when coupled with a change in environment. [110,162,169,172,173] While shown to be effective, administration of GCs is not without unwanted side effects [209] such as adrenal suppression [171], and the potential for laminitis and immunosuppression predisposing to increased infections. [178] When given to human asthmatics, the GC dose and frequency of administration are gradually decreased to minimize the risk of adrenal suppression and establish the lowest and least frequent dose required to achieve the desired clinical effect. Extrapolating from human medicine, a similar approach to GC therapy is used in the treatment of equine heaves. However, the effectiveness of this approach has not

been well explored in any species, and it is not known whether resolution of clinical disease consistently correlates with resolution of airway inflammation as dose amount and frequency decreased. In addition, the effect of tapering GC therapy on inflammatory signaling events has also not been explored.

The nature of the inflammatory response has been well studied in human asthmatics and there is a large body of evidence to indicate that a Th2 type immune response is associated with the development of this disease. This response is characterized by an increased expression of IL-4, IL-5, IL-6, Il-10 and IL-13. It is not clear whether this association applies to horses with RAO, since studies aimed at measuring cytokine expression in airway inflammatory cells retrieved from horses during both remission and crisis have reported conflicting results. In fact, some investigators suggest that a Th1 response may predominate in chronically affected RAO horses based on an increased expression of IFN-γ detected in airway inflammatory cells. The effect of glucocorticoids therapy has also been minimally explored, and it is not known whether glucocorticoids induced alterations in cytokine expression are changed by tapering the dose or extending the dose interval of glucocorticoids.[87]

To better define the response of RAO affected horses to tapering dose GC therapy, we used a three way cross over design to evaluate and compare the response of 6 acutely affected RAO horses to three commonly used treatments for RAO. The treatments included: environmental modification (ENV) with saline placebo; a tapering dosage protocol of dexamethasone (GC) intravenously (DEX); or a combination of both treatments (DEX + ENV). Treatments were administered for a period of 22 days and clinical parameters, BALF cytology, pulmonary lung function tests and BALF cytokine expression of IL-4 relative to INF-γ expression was measured before and throughout the treatment period. Our findings indicated that changes in clinical parameters and pulmonary function did not differ significantly over time between treatment groups. In addition airway neutrophilia resolved within 3 days of a change in environment, and this response was not affected by the addition of glucocorticoids to the horse's treatment. In contrast horses receiving dexamethasone in the absence of environmental change appeared clinically improved over the 22 days of treatment – but showed less of a decline in airway neutrophilia throughout the study and experienced a significant rise in the percentage and total number of BALF neutrophils when treatment interval was extended to 72 hours. Changes in airway neutrophilia coincided with a change in the ratio of IL-4/ IFN-γ expression in BALF cells, but a similar relationship was not observed in those horses treated with dexamethasone, with or without environmental change. In fact the ratio of IL-4/ IFN-γ was lowest in the DEX group at the sample time in which the BALF neutrophil percentage was the highest (day 22). These findings suggested that the mechanism by which dexamethasone induced clinical improvement in RAO affected horses was not identical to the mechanism that reduced airway inflammation, and the reduction of airway inflammation may have required a higher and or more frequent dosing regimen than that which was required to provide clinical improvement. In addition, dexamethasone induced resolution of airway neutrophilia may not be related to a

predominant Th2 cytokine response. These findings indicated that at longer dosing intervals, a rebound airway neutrophilia may have occurred in the absence of recurrence of clinical disease. Therefore determination of the lowest effective dose of dexamethasone should include evaluation of BALF cytology to be certain that airway inflammation as well as clinical signs is fully resolved. In addition, airway neutrophilia resolved by the third sample period (day 60 but returned by day 22 while the relative expression of IL-4 to IFN-γ by BALF cells continued to decline throughout the experimental period. These findings indicate that if the Th2 and Th1 paradigm applies to equine RAO, then the cytokine mediators of airway inflammation may differ from those that influence clinical parameters in RAO affected horses treated with dexamethasone alone. The results of our findings provide additional evidence that environmental change is a critical component of RAO management. In addition, we discovered that horses receiving low dose dexamethasone every third day may appear clinically improved, however they may continue to display significant airway inflammation.

Materials and Methods

Horses and Housing Conditions: Six adult RAO affected horses comprising 2 mares and 4 geldings and ranging in age from 11 to 24 years were selected for the study. These horses originated from a pre-existing herd (RAO herd) that is maintained at Virginia Tech and met the criteria specified for inclusion into the RAO herd, developed clinical signs of RAO in challenge environment and entered clinical remission when returned to pasture. The horses were maintained on pasture for a minimum of 16 weeks prior to the study.

The horses were housed in three different conditions:

- Pasture: Grass pasture with access to free choice water and mineral supplement (salt block).
- Challenge Environment: Individual 12 x 12 foot stalls in a research barn, bedded with straw. Horses were fed dusty hay during this period to induce clinical disease. They were allowed a 1 hour turnout period per day.
- Remission Environment: Horses were housed in a 12 x 12 foot stall, bedded on low dust wood chips, and fed a complete pelleted fed with continuous access to a 12 X14 foot outdoor pen adjoining the stall

Study Design: *see table 2.*

The 6 study horses were brought from the pasture into stalls within a barn. Each stall was prepared using straw as bedding and dusty hay as feed. The horses were housed in this challenge environment in order to induce signs of RAO. Sufficient evidence of clinical disease was considered to be a clinical score of $\geq$ 5 or ΔPpL_{max} >15 cm H_2O). At that time pulmonary function testing and a bronchoalveolar lavage were performed on all the horses. This was considered to be sample 1. After sample collection, horses were allocated into treatment groups. A replicated 3-period, 3-treatment crossover design, balanced for residual effects, with repeated measures, was used for this study. **Group 1** remained in the challenge environment and received

dexamethasone in tapering doses (DEX), 0.1mg/kg, IV, q 24 h for 3 days followed by 0.05mg/kg, IV, q 24 h for 3 days, then 0.05mg/kg, IV, q 48 h for 3 treatments, then 0.05mg/kg, IV, q 72 h for a further three treatments for a total treatment period of 22 days. **Group 2** was housed in the remission environment and treated daily with an amount of intravenous sterile normal saline equal to that required to deliver the dose of dexamethasone appropriate for that treatment day (ENV). **Group 3** horses received dexamethasone, in tapering doses, as described in table 2 and were housed in the remission environment. Since housing RAO affected horses in a challenge environment for 22 days without treatment is considered inhumane by this research group we did not evaluate an untreated group. Samples 2-5 were collected at days 3, 6, 12 and 22 of treatment respectively. The time period between samples was not constant as the duration between treatments was gradually increased. Relative to delivery of the third dose, samples were taken at the end of the presumed effective period for each dexamethasone dose and interval. This design permitted us to determine if the effect of treatment persisted to the end of the interval between treatments.

Samples were taken after horses had been treated three times with either dexamethasone at the appropriate dose and interval or the equivalent amount of saline. For example, when horse received daily dexamethasone at a dose of 0.5 mg/Kg, these horses were sampled 24 hours after the third does was administered. Likewise, samples were taken from horse 48 hours after the last of three doses of dexamethasone, 0.05 ml/Kg IV every other day was administered. This approach was chosen to permit evaluation of the period of time each tapering dose was expected to have an effect. Horses were not treated until after all samples were collected. A washout period of approximately 14 days between treatments was used. Each sample point included a physical examination, clinical scoring for nostril flare and abdominal push as describe by Rush [126] (*see table 1*), pulmonary function testing; and collection of bronchoalveolar lavage fluid (BALF) for cytological analysis. BALF from samples 1, 3, and 5 were also evaluated for cytokine expression (IL-4 and IFN-γ). Samples were collected before treatment was administered.

Clinical Evaluation and Lung Function Testing During Spontaneous Breathing: *see appendices for full methodology.*

A complete physical examination included rectal temperature, heart rate, respiratory rate, evaluation of hydration and gut motility, heart and lung auscultation. Clinical scores were allocated as previously described (*see table 1*). Nasal flare and abdominal push scores were subjectively evaluated according to previously described methods. [128,152] Nasal flare was evaluated by assessing the extent of deviation of the alar cartilages from normal resting position. Abdominal push was the degree of involvement of the abdominal musculature in expiration. A scale of 1 to 4 was used with a grade of 1 being normal and grade 4 being most severely affected. The clinical score was the cumulative score of the nasal flare and abdominal push scores.[152]

Pulmonary function testing was performed on unsedated horses prior to the bronchoalveolar lavage procedure. Pulmonary function testing was performed by passing an esophageal balloon (10cm long, 3.5cm perimeter, 0.06cm wall thickness) sealed over the end of a polypropylene catheter (3mm internal diameter, 4.4mm external diameter) into the distal third of the esophagus. This was attached to a very low range differential pressure transducer. The position of the esophageal balloon was adjusted to obtain the maximal changes in pleural pressure during tidal breathing (ΔPpl_{max}). Respiratory flow was measured using a pneumotachograph connected to an airtight face mask and coupled to a differential pressure transducer that provided a signal proportional to flow. Flow and pleural pressure during breathing were processed by a lung function computer using the BUXCO physiographic system and software (BUXCO Electronics, Torrington, CT) to calculate tidal volume , pulmonary resistance, dynamic compliance, ΔPpl_{max}, respiratory frequency, inspiratory and expiratory time (mean inspiratory and expiratory flow. At each data collection time, values of at least 30 consecutive breaths were averaged.

Bronchoalveolar Lavage Fluid (BALF) Collection and Cytology: *see appendices for full methodology.*

Horses were sedated with intravenous detomidine [2] (0.01 mg/kg) and butorphanol[3] (0.01 mg/kg). A bronchoalveolar lavage tube (Bivona) was passed through the nasal passages and into the trachea. A dilute lidocaine solution (0.4%) was infused and the tube was advanced until it wedged in a distal airway. The bronchoalveolar lavage tube balloon was inflated. Four aliquots of 60 ml 37°C sterile saline were infused and aspirated. The recovered BALF volume was recorded. Within 20 minutes after collection, the BALF was centrifuged at 200 x g for 15 minutes at 4˚C. From the cellular pellet, the total nucleated cell count was determined. A cytospin machine was used to prepare a slide for cytological analysis with a portion of the fluid. The slide was stained using a modified Wrights stain. The differential cell count was evaluated by counting 200 cells. The primary investigator evaluated all the slides while two of the other investigators reviewed 20 slides to compare results and ensure accuracy. The results of the primary investigator were used in statistical analyses.

Semi quantitative measurement of mRNA expression of IL-4 and INF-γ expression in BALF cells (Samples time 1, 3, and 5).

BALF cells were collected for analysis of gene expression to compare expression of IL-4 and IFN-γ during the S1, S3 and S5 sample periods of this study. These sample times were chosen as it was considered the best potential period to demonstrate a difference between and within the RAO-affected and control horse groups.

Analysis of cytokine expression in airway lymphocytes was performed on cells harvested after centrifugation of BALF and subsequent storage in 1 ml RNA*later*® (Ambion, Inc; Auston, TX) at -80^{o} C (*see appendices for full methodology*). These cells were then thawed and prepared using QIAGEN RNeasy[4] protocol in order to isolate the mRNA prior to RT-PCR[5]. The iQ SYBR

Green Supermix Reagent System (Invitrogen; Carlsbad, CA) was used following the manufacturer's instructions. cDNA amplification was achieved using the following primers:

CYTOKINE	SENSE PRIMER	Tm(◦C)	ANTISENSE PRIMER	Tm(◦C)
IL-4	AATGCCTGAGCGGACTG	55.5	TGCTCTTCTTGGCTTCATTC	54.1
INF-γ	TGAAGGTCCAGCGCAAAGC	59.5	CTGACTCCTCTTCCGCTTCC	57.9

Real-time amplification was performed using a 170-8740 iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Specific annealing temperatures were set for each cytokine based on the optimal annealing temperature determined by the temperature gradient analysis protocol *(see appendices).* The plates were set up with all the standards for one cytokine contained in each plate. The standards were run as triplicates. Data was log transformed (ln) and expressed as a ratio of start quantity of IL-4 to start quantity of IFN-γ.

Statistical analysis – All analysis was performed using SAS version 9.2 (Carry NC, USA). For all data except the cytokine expression of IL-4 and IFN-γ, a Mixed-model repeated measures of analysis of variance was performed to compare post treatment samples to pretreatment (baseline) samples in order to demonstrate the effect of each treatment. The same method was used to compare the three treatments in order to determine the best treatment option. Mixed model repeated analysis of covariance was used to compare the samples during the course of treatment.

Results

Clinical parameters *(see table 3, figure 4a; 4b; 4c)* **–** Rectal temperatures remained stable throughout the study period. Heart rates in both the DEX, and the ENV groups did decrease from sample period 1 (crisis) through to the sample period 4. This change was statistically significant in both groups, but of little clinical relevance because parameters remained well within clinically normal limits. The horses receiving both dexamethasone and environmental modification did not demonstrate a consistent decrease in heart rates. Respiratory rates in all three treatment groups decreased from the crisis period in response to treatment. Horses in the group receiving dexamethasone treatment without environmental modification demonstrated a non-significant increase in respiratory rates from sample period 3 (0.05 mg/kg dexamethasone IV q 24 h) to sample period 5 (0.05 mg/kg dexamethasone IV q 72 h). Abdominal push scores decreased in response to treatment in all three treatment groups (*figure 4a*). There was a significant difference in the abdominal push scores at sample period 5 between the DEX group (mean 2.2) and the DEX + ENV group (mean 1.7), and between the ENV group (mean 2.1) and the DEX + ENV group (mean 1.7). Nasal flare scores decreased from sample 1 (crisis) in response to treatment in all three groups but was not significant (*figure 4b*). Total clinical scores decreased in response to treatment in all three groups, but only the DEX + ENV group was statistically significantly

different between sample 1 (crisis) and samples 4 (0.05mg/kg dexamethasone IV q 48 h) and 5 (0.05mg/kg dexamethasone IV q 72 h) (*figure 4c*).

Pulmonary function testing during tidal breathing *(see table 4, figure 5a; 5b; 5c)* **–** All RAO affected horses had an elevated ΔPpl_{max} during the crisis period consistent with airway obstruction. By comparison mean ΔPpl_{max} decreased in all three treatment groups in response to treatment (*figure 5b*). However these changes were not statistically significant. The DEX group significantly increased between samples 4 (0.04 mg/kg dexamethasone IV q 48 h) and 5 (0.05mg/kg dexamethasone IV q 72 h). Dynamic compliance (C_{dyn}) tended to increase in all horses at the start of the treatment period. The DEX + ENV group increased significantly during the treatment period with sample period 4 and 5 being statistically increased compared to pretreatment values (*figure 5a*). Statistically there was a significant deterioration in C_{dyn} from sample 3 (0.05mg/kg dexamethasone IV q 24h) to 5 (0.05 mg/kg dexamethasone IV q 72h) in the DEX group. All three treatment groups had increased tidal volume (TV) initially followed by a decrease towards the end of the treatment period (*figure 5c*). The DEX group had a significantly increased tidal volume compared to the ENV at sample 3. Inspiratory tidal volume (T_i) was below normal in all three treatment groups during the crisis period and increased in response to treatment in all three groups. The response to treatment demonstrated by a mean increase in inspiratory tidal volume in both the DEX + ENV group and the ENV group at sample 2 was significant. Dexamethasone treatment without environmental modification resulted in a significant decrease in T_i as the interval between dexamethasone treatments increased from every 48 hours to every 72 hours. The T_i at the end of the 21 day treatment period was significantly different between the DEX group compared to the DEX + ENV group. The mean expiratory tidal volume (T_e) was non-significantly increased in all three groups prior to treatment. No consistent response was seen between groups. The DEX group and the DEX + ENV group differed significantly at the end of the treatment period with the DEX group being closer to expected normal values. Pulmonary resistance did not differ significantly between treatment groups or sample times.

Bronchoalveolar lavage cytological evaluation *(see table 5, figure 6a; 6b; 7a; 7b; 8a; 8b; 9a; 9b; 10)* **–** Comparison between investigators revealed a good correlation between cytological differentials, with a BALF neutrophil count median of 2 cells (range of 0-11 cells). There was no significant difference in the mean BALF volume and mean total cell counts between sample periods and treatment groups. Macrophage and lymphocyte cell counts did not change significantly throughout the treatment period in any group with the exception of the DEX group where lymphocyte percentages decrease as neutrophil percentages increased (*figure 7a; 7b; 8a; 8b*). However, absolute numbers of lymphocytes were unchanged. The percentage of neutrophils in the BALF was initially increased in all three groups prior to treatment (*figure 9a*). The ENV group and the DEX + ENV group demonstrated a decrease in airway neutrophilia in response to treatment which was maintained throughout the 22 treatment period. The DEX group initially showed a decrease in the percentage of neutrophils within the BALF but this was followed by a

significantly increased BALF neutrophil percentage that was most notable between the administration of dexamethasone at 0.05mg/kg IV every 48 hours and the administration of dexamethasone at 0.05mg/kg IV every 72 hours. At the end of the 22 day treatment period there was a significant difference between the DEX group compared to the other two treatment groups. There was no significant difference between the ENV group and the DEX + ENV group in terms of percentage neutrophils in the BALF at the end of the treatment period.

BALF cytokine expression – No significant change in the relative expression of IL-4 to IFN-γ occurred during the sample periods in which cytokine expression was measured. However, the ratio declined throughout the treatment period in the DEX treated group and was unchanged in the DEX + ENV treated group. In the horses treated with environmental modification alone (ENV), the ratio declined between sample period 1 and 3 during which time the horses demonstrated improvement in clinical signs and a reduction in airway neutrophilia. Thereafter the ratio tended to increase between sample 3 and 5, which correlated with a slight (but insignificant) decline in pulmonary function and an increase in airway neutrophilia.

Discussion

Following exposure of the horses to a challenge environment all horses in the study demonstrated clinical signs typical of RAO affected horses when exposed to inhaled allergens. Clinical parameters including heart rate, respiratory rate, nasal flare and abdominal push scores were consistent with these signs. In response to treatment all horses demonstrated decreased respiratory rates, nasal flaring and abdominal effort which were consistent between treatment groups. These changes, in some cases, were statistically significant but were not generally considered clinically significant since the mean values of these parameters were within the normal range. This finding suggested that subtle changes in clinical parameters overtime in individual animals may be more meaningful when attempting to detect evidence of recrudescence of disease, while application of broader reference range values may obscure changes that signify return of disease in RAO affected horses.

Scores of abdominal push (APS), nostril flare (NFS) and their sum (total clinical score or TCS) generally did not significantly change through the study period. However, APS was significantly lower in the DEX/ENV groups as compared to the other treatment groups towards at sample 5, and APS and TCS differed significantly from the mean pretreatment scores at the same sample times. This finding may have indicated that the combination of environmental change and dexamethasone was a superior treatment, especially as the dose of dexamethasone was reduced to an every third day treatment. It was not known how long the immunomodulatory effects of dexamethasone administration persist, but daily or twice daily treatment has been recommended in horses for the purpose of immune suppression.[210] Previous reports using an in vitro system indicate that cytokine expression may have remained attenuated for as long as two days after administration of intravenous dexamethasone[211] indicating that immune attenuation persisted beyond the period of expected therapeutic concentrations. However clinical scores have been

compared to more objective measures of pulmonary function, and shown to be an insensitive method for detecting disease in RAO horses that are not severely affected. [131] These results supported that conclusion since little change was noted between the pretreatment and early treatment values in all treatment groups, even though all horses met our criteria (clinical score of >5 or ΔPpL_{max} >15 cm H_2O).

In all treatment groups, the mean values for ΔPpl_{max} and C_{dyn} decreased while tidal volume increased after treatment was initiated. Significant changes were detected in later sampling periods in the DEX treated group. At sample 3 (treatment day 6), horses treated with DEX displayed evidence of significant pulmonary function improvement, as indicated by an increase in C_{dyn} and TV. This response indicated peak improvement in pulmonary function at day 6, when horses were receiving 0.05mg/Kg IV of dexamethasone daily. When dosing was extended to every other day, TV declined significantly by the next sample time (Sample 4, day 12). Following every three day treatment (Sample 5, day 21), a further decline in pulmonary function was indicated by a significant decrease in ΔPpl_{max} as compared to Sample 4: a significant decrease in C_{dyn} as compared to Sample 3, and a significant decline in TV as compared to Sample 3. The results indicated that horses treated with DEX experienced persistent, albeit reduced, pulmonary dysfunction when dexamethasone treatment intervals were extended beyond twenty-four hours. Thus dexamethasone, in the absence of environmental modification, may not be an effective treatment of RAO unless the dosing interval remains therapeutic. This compromise, while detectable through pulmonary function testing, was not obvious using parameters of the general physical examination.

In contrast, horses treated with DEX + ENV demonstrated improved pulmonary function through Sample 4 (day 12 or every other day dosing) with a mild but statistically significant reduction in function when dosing was extended to every third a day. As compared to the DEX only group, this outcome indicated that environmental modification coupled with every other day dexamethasone administration was an effective treatment for RAO. Long-term dexamethasone administration has been recognized as a cause of iatrogenic hypoadrenocortism. While this association has not been identified in the horse, intrinsically, one might predict that every other day dosing of dexamethasone may pose less risk of adrenal suppression, especially in horses requiring long term therapeutic management of RAO.

The BALF volume and total cell number in the fluid did not vary significantly with treatment or sample. The percentage of macrophages and total macrophages per sample were also not affected by treatment or sample time. In all cases, horses experienced a mild to moderate neutrophilia when sampled prior to initiation of treatment (Sample 1). Horses in the DEX + ENV and ENV group demonstrated reduced airway inflammation once treatment was initiated, as confirmed by a reduction in neutrophil percentage and total numbers. However, the percentage and total neutrophil number trended towards being higher throughout the treatment period in the DEX group as compared to all others. The finding that airway neutrophils were significantly higher in the DEX group as compared to the DEX + ENV and the ENV groups at all sample times except

4 indicates that although clinical parameters and pulmonary function was generally improved in the DEX horses during most of the treatment time, airway neutrophilia failed to fully resolve. Horses in the DEX group also experienced a rapid and severe rise in airway neutrophil percentages and numbers as treatment frequency was reduced to every third day.

These findings suggested that low grade persistent inflammation placed these horses at risk for more acute, severe recrudescence of inflammation as compared to those animals that experienced a period of inflammatory resolution as observed in the ENV and DEX + ENV groups. These findings emphasized the need to monitor airway cytology as well as clinical parameters when trying to determine the lowest effective dose and longest effective interval of dexamethasone administration. In addition, the marked difference in airway neutrophil counts in horses whose treatments included environmental modification as compared to those that remaining in the challenge environment confirmed the importance of environmental modification in the treatment plan regardless of corticosteroid administration. The phenomenon of significant increases in BALF neutrophil proportions in the dexamethasone treatment group towards the end of a 21 day course of treatment also deserves further study. This did not appear to be as a result of the corticosteroid medication as this response was not seen in the group treated with corticosteroids and environmental modification. BALF eosinophil counts remained insignificant throughout the entire study confirming previous reports of minimal eosinophilic involvement in equine recurrent airway obstruction, unlike in human asthma. Likewise, only a small percentage of horses had mast cells or basophils in their BALF, and when these cells were identified, they were present in very low numbers (1 - 2%)

This finding indicated that the use of dexamethasone in the treatment of acute RAO was effective in reducing airway inflammation and improving clinical signs but that without modification of environmental conditions airway inflammation will rapidly return. The horses demonstrated subtle changes in clinical parameters during this time period, however clinical assessment was not a sensitive indicator of these changes and it is unlikely that they will be appreciated by owners. While parameters of pulmonary function were a more sensitive indicator of disease recrudescence, these measurements cannot currently be performed by most equine practitioners or horse owners. Thus, the management of RAO affected with dexamethasone only may result in persistence of airway neutrophilia despite clinical improvement, and the persisting inflammation could possibly play a role in airway remodeling. Further studies are needed to investigate the long term effect of ongoing airway inflammation in horses remaining in the challenge environment.

Expression of cytokines associated with CD4 Th2 lymphocyte phenotype is considered a component of the pathogenesis of human asthma. Reports concerning the balanced cytokine expression in airway cells from RAO affected horses have not provided a consistent picture and clear understanding of the role of Th1 and Th2 lymphocytes in this disease. Using RT-PCR, we measured the relative gene expression of IL-4 to INF-γ as markers of relative Th2 to Th1 gene expression in airway cells retrieved at sample time 1, 3, and 5, respectively. These results did not

suggest a clear relationship between measures of severity RAO and cytokine expression. When the IL-4/INF-γ ratio was plotted concurrently with neutrophil counts again, no clear or consistent relationship was detected although trends as described in the results section were observed. The absence of a definitive relationship between IL-4 and IFN-γ expression and resolution of airway neutrophilia may be due to several factors. Firstly, in our analysis, lymphocytes were not separated from other cells in the BALF. While IL-4 is expressed by CD4 Th2 lymphocytes, the initial cell source of IL-4 which serves to promote Th2 development has not been identified. IL-4 production has been detected in a broad array of cells ranging from human amnion epithelial cells to insect NK cells, so potential production by one of the other cell populations in BALF is possible.[212,213] Likewise INF-γ is expressed by cells other than Th1 lymphocytes, dendritic, natural killer cells (NK) and Tc. The potential presence of these cells in our sample may have influenced the amount we measured. Previous studies have also shown that dexamethasone therapy suppresses peripheral blood lymphocyte expression of both IL-4 and INF-γ. The data coupled with the responses in our study, suggested that IL-4 and INF-γ expression may not contribute to the establishment of RAO during the acute phase of the disease, and dexamethasone induced reduction in either or both of these cytokines may not have a measureable effect on the parameters we evaluated in our study.

In conclusion, the three treatments all appear to be effective in improving clinical parameters, pulmonary mechanics and airway inflammation associated with an acute RAO crisis. Both environmental modification and a combination of environmental modification and a decreasing dexamethasone treatment protocol are effective in reducing clinical compromise and airway inflammation and maintaining this effect for the full 21 day treatment period. In contrast, horses treated with only dexamethasone had persistent evidence of functional compromise and airway inflammation which drastically worsened as the frequency of steroid administration became prolonged. These results illustrate the importance of the inclusion of environmental modification in the long-term management of RAO. While tapering doses of dexamethasone without environmental changes was an effective treatment while on the higher frequent dosing schedule, the results of this study demonstrated that airway inflammation can persist when the dose of dexamethasone is reduced, and in the absence of clear clinical evidence of compromise. Significant airway inflammation in the absence of clinical manifestation of disease may be related to the change in pulmonary tissue morphology that is observed in horses with chronic heaves. Further investigation of this effect in human asthma patients is warranted.

Footnotes

Equine Senior[1], Triple Crown, Southern States
Detomidine[2], Pfizer Exton, PA
Butorphanol[3], Fort Dodge, Fort Dodge, IA
QIAGEN RNeasy mini kit plus[4], USA
170-8740 iCycler iQ Real-Time PCR Detection System[5], Bio-Rad Laboratories, Hercules, CA.

Chapter 3

Discussion

A comparative study of three of the commonly used treatments for RAO in horses is clinically useful and justified. Choosing these three treatment protocols stems from the approach by equine clinicians in the field as they are easy to do and appears to be effective. Each of these treatment approaches has advantages and disadvantages which may determine selection in different situations.

Several of the discoveries that resulted from this study have direct clinical application. The horses used in this study were selected from an established RAO herd. Each horse had therefore been previously evaluated for the condition according to a known set of parameters. The horses selected were required to show a quick onset of typical RAO clinical signs when exposed to the chosen challenge environment and then rapidly resolve when placed in a paddock. This is important because the horses used in the study responded to challenge within an expected time period thereby minimizing individual variation in onset of treatment. As a result treatment and sample collection could be performed as a group which eliminated variation due to daily fluctuation in ambient temperature, humidity, etc. The horses of the RAO herd were well accustomed to the various procedures including pulmonary function testing and bronchoalveolar lavage. This eliminated the need for sedation in order to measure pulmonary function. In addition the release of endogenous corticosteroids caused by stress was minimized and interference with treatment protocols reduced. Ample help in the form of experienced technical staff and student helpers was also available which allowed us to develop efficient, standardized methods for executing our procedures.

The barn design was ideal in that the entire study could be performed in the barn without the need to move horses from their environment in order to perform procedures. This minimized the effect of change in environment, treatments, and evaluations on the parameters that we measured.

A replicated three-way, three treatment crossover design that was balanced for residual effects was used. This method minimizes the impact of individual variation by randomizing the sequence in which horses receive treatments such that all combinations were equally applied. This minimized potential bias or residual effects of treatment. A suitable washout period was also included to allow for complete separation of treatment effects.

Multiple parameters were assessed in the study including clinical findings, BALF cytology and cytokine expression, and pulmonary lung function testing. This permitted efficient use of the horses and provided the best opportunity to examine the effect of the treatments used in this

study. BALF cytology was analyzed blindly to avoid bias. The cytological findings were also confirmed by three of the investigators to ensure their accuracy.

Among the most important findings in the study were that moderate environmental changes resulted in significant improvement in clinical status of RAO affected horses. The improvement corresponded with reduced airway inflammatory cells and neutrophil numbers. The coupling of dexamethasone treatment with environmental modification did not dramatically change the outcome. Dexamethasone treatment did diminish signs of clinical disease however the reduction in BALF neutrophil numbers was not as significant. The discovery of increased BALF total neutrophil counts and percentage neutrophils as drug administration was decreased to every third day was vital. This finding suggested that airway inflammation returns as the interval between dexamethasone dosing is extended to 72 hours in horses where environmental modification was not coupled with the treatment. The increased BALF neutrophilia seen towards the end of the dexamethasone treatment was not accompanied by significant clinical deterioration indicating that horses treated with dexamethasone may experience a significant airway neutrophilia without clinical signs of disease. While the long term significance of persistent airway neutrophilia has not been examined in the horse, it is possible that low grade tissue damage results and contributes to the chronic deterioration in horses with this disease. The DEX + ENV treatment was not noticeably superior to ENV alone; however the DEX + ENV group did demonstrate the most improvement in C_{dyn} between sample period 3 and 5. This may be consistent with the combination of treatments being more effective at restoring normal characteristics of lung tissue that contribute to compliance.

The study was strengthened and variability reduced by the use of a three-way cross-over design, selection of horses with similar disease characteristics, and application of well-defined, controlled treatments and environments. However, the study could have been improved in a number of ways:

1) Only 6 horses were used in the study in a 3-way cross-over design. This number of horses ensures that the results obtained from the study are valid and minimizes the cost of the study. Including a greater number of horses would have strengthened the reliability of the results as this would minimize the influence of individual variation.

2) Evaluation of horses at more frequent intervals could have been performed. This would be especially relevant in the group receiving dexamethasone alone. The airway inflammation returned as the interval between treatments increased, but it is not known whether this change occurred when the dose interval was extended to every two or every three days. More sample periods during this time would be advantageous to more accurately determine the changes occurring in the airways during this time.

3) Determination of the dosing interval at which the majority of horses remained in remission is a clinically important and was not determined in this study.

4) Extending the length of the study beyond the 21 day treatment period is recommended. The majority of changes in airway inflammation began to occur near the end of the treatment period suggesting that difference in treatment response may have become evident if the protocols were evaluated for an additional period of time. It would be useful to determine the effect of treatment beyond this time period as well as the onset of clinical signs in relation to these changes. Further investigation into the persistence of airway inflammation is needed along with long term studies on airway remodeling and whether the two conditions are associated.

5) The environment used in this study as the challenge environment consisted of the addition of straw bedding and dusty hay to the stable environment. This is by no means an extreme challenge environment. The barn setup for comparison of the selected treatments was adequate but had the following limitations. Firstly the horses receiving different treatments were housed in fairly close proximity. Based on work by Clements et al.[163] it has been shown that adjacent stalls can impact the environment in the selected stall. This is of importance in this study as the environmental modification was a vital part of the study and without adequate prevention of cross-over contamination from surrounding stables the response to treatment may be more difficult to assess. The treatment with environmental modification was likely less effective due to the persistence of allergens in the environment from surrounding stables. Potentially including a method of measuring airborne particles in the study may have allowed a direct comparison of the exact allergen load that the horses were exposed to.

6) The use of intravenous administration in this study was selected to guarantee that the complete dose was received by the horse. Oral administration is affected by feed intake and absorption and would therefore have made analyses more challenging. However, oral administration is commonly used in horses with RAO as most owners are not able to inject the medication intravenously and intramuscular administration can lead to abscessation. The results of this study may be more readily adapted to clinical practice if the more common, client based method of oral administration was used to deliver the dexamethasone.

7) Further assessment of the impact of dexamethasone administration on immune function would also have provided valuable information about the management of clinical aspects of RAO. BALF cell cytokine expression was included in the study to aid in determination of the cytokine profile present in the BALF during various stages of RAO. However the cytokines were only evaluated at three of the 5 sample times due to financial constraints of the project. Further studies into the cytokine response to treatment evaluating a wider cytokine profile as well as those potentially more involved in airway remodeling are studies for the future.

Findings from this study lead to other potential investigations that could be performed. These include the evaluation of different phases of RAO by comparing acute versus more chronically affected horses and potentially determining the prognosis for response to treatment. More complete analysis of the stable environment is needed to determine the response to each environmental change. This may allow owners to make small changes that result in the greatest clinical relief for their animals. Different stable designs have been evaluated for aspects of ventilation and airborne load but this aspect could potentially also be used as a comparative treatment option - movement of horses to a different barn design and eliminate the need for additional treatments. Other treatment regimens can be evaluated including different routes of treatment, regimens of corticosteroids or a combination of corticosteroids and bronchodilators. Similar drug combinations to those used in human asthma could also be compared to those evaluated in this study.

In summary the results of this study demonstrated that the clinical response of RAO horses was not significantly different such that one treatment was clearly superior to another. However treatments differed significantly in their ability to attenuate airway inflammation. Dexamethasone treatment, in the absence of change in environment was the least effective method for minimizing airway inflammation, and horses receiving this treatment may experience significant airway inflammation in the absence of detectable clinical compromise. This study raised the question of the cause of increased inflammation in the airways despite corticosteroid medication at the end of the treatment period. This effect can be explained by the persistence of environmental challenge and the waning affectivity of the corticosteroids which leaves the horse's immunity to respond to inciting agents resulting in an increase in inflammation. This makes sense, but leads us to believe that an RAO horse maintained in a challenge environment while on corticosteroid medication may continue to experience airway inflammation without clinical evidence of deterioration. This is important in that persistence of inflammation could be involved in structural changes that take place within the airways leading to irreversible pulmonary fibrosis. This will likely limit the horse's athletic usefulness and response to therapy. This concept is one which should be further evaluated – both for the treatment of RAO and for human asthma. Many human asthma sufferers are maintained for life on inhalant medications which contain corticosteroids, often in combination with other medications. It is well known that chronic inflammation can lead to fibrosis and scar formation, and the horse may prove to be a good model for evaluating treatments aimed at minimizing these effects. Reducing airway inflammation would be an important step in preventing the onset of remodeling. This would prolong human asthma sufferers lives and RAO horse's competitive careers.

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Appendices

A. Clinical score

The system described by Rush et al. (1998) was used. Nasal flaring and abdominal push movements were both scored on a scale of 1 to 4. *See table 1 for a description of scoring.* The results of the two scores were added together to give a total clinical score. A total clinical score of 2 represented no clinical signs while a score of 3 or 4 was consistent with mild signs, 5 or 6 with moderate signs and 7 or 8 severe signs. Clinical scores were determined prior to any procedures. The clinical scores were performed by technical help and checked by a veterinarian. All evaluators were blinded to the treatment that each horse received, but was not blinded.

B. Pulmonary function testing

The procedure was performed in horses during normal tidal breathing without sedation. An esophageal balloon catheter (10cm long, 3.5 cm perimeter, 0.06cm wall thickness) was passed into the distal third of the esophagus followed by placement of an airtight mask over the horse's muzzle and nostrils. A pressure transducer (Validyne model DP/45-28, Validyne Engineering Corp, Northridge, CA) was attached to the esophageal catheter and measured changes in pressure in the esophagus during peak inspiration and expiration while a flow transducer (Buxco Electronics Inc, Wilmington, NC) present in the nasal mask measured air flow. The position of the esophageal catheter was adjusted to obtain the maximum change in pleural pressures (ΔPpl_{max}) during tidal breathing. The flow and pleural pressure changes were processed by a pulmonary function computer using the BUXCO pulmonary function software (Buxco Electronics Inc, Wilmington, NC) to calculate tidal volume (V_t), pulmonary resistance (PL), dynamic compliance (C_{dyn}), respiratory frequency (f), inspiratory and expiratory time (T_i and T_e, respectively) and Resistance (R_L). At each data collection time, values of at least 30 consecutive breaths were averaged.

C. Bronchoalveolar lavage

1. Horses remained in their stall environment.
2. Each horse was sedated with intravenous detomidine (Pfizer Exton, PA) (0.01 mg/kg) and butorphanol (Fort Dodge, Fort Dodge, IA) (0.01 mg/kg).
3. A period of approximately 5 minutes was allowed to ensure adequate sedation was obtained.
4. The horse's nostril was lubricated with a small amount of lubricating gel and lidocaine mixed using approximately 10 ml 2% lidocaine with 20ml lubricating gel.

5. Once the sedation took effect, the horse's head was elevated to allow the muzzle and trachea to form a straight line.
6. A 3 meter bronchoalveolar lavage tube (Bivona, Gary, IN) was passed blindly through the horse's nasal passages and into the trachea.
7. A dilute lidocaine solution (0.4% lidocaine) was infused through the tube prior to further passage to reduce the cough reflex.
8. The tube was then advanced down the trachea until it wedged in a distal airway.
9. The balloon was then inflated using 6ml of air.
10. Four aliquots of 60 ml sterile saline (37°) were infused and re-aspirated.
11. The aspirated fluid was mixed and pooled in a sterile specimen cup maintained on ice.
12. Recovered fluid volume and quality (ie. amount of foam, presence of mucus) were recorded.
13. Visible strands of mucus were removed with a pipette prior to further processing.
14. Samples were processed within 30 min after collection in order to minimize sample degradation.
15. Total and differential cell counts were performed.
16. BALF was centrifuged at 400 x g, 4°C, for 10 minutes to pellet cellular components.
17. The cell pellet was washed twice with DPBS and re-centrifuged. The cell pellet was resuspended in 1-2 mls of DPBS. A portion was used to perform a total cell count using the Casy 1 cell counter. A small amount was used for cytological assessment following cytospin preparation; The remainder was divided into smaller 1.5ml micro-centrifuge (sterile RNase/DNase free) with 5 x 10^6 cells/tube.
18. 10mls of sterile DPBS along with 10µl of cells were added to each vial used in the determination of total cell count using the Casy 1 cell counter.
19. The tubes are spun in a bench top centrifuge (2 mins. At 1400rpm) and the supernatant is aspirated.
20. 1 ml of RNA*later*® (Ambion, Inc; Auston, TX) was added to the sample. The sample was homogenized by passing the lysate through a 20g needle attached to a 1cc syringe at least 5 times.
21. The tubes were appropriately labeled and immediately frozen at -80°C until analyzed.

D. Cytological evaluation

1. The second portion of cell suspension was used to prepare a cytospin slide for differential cell analysis.
2. 50µl 30% bovine serum albumin was placed in the sample chamber funnel.
3. The sample was then diluted based on the total cell count obtained in the Casy 1 (Casy 1, Scharfe system). The sample was diluted using DPBS. Ideally the number of cells loaded in the cytospin should equal approximately 100,000 cells.

4. 200µl of cell suspension was added to the sample chamber.
5. The cytospin (Shandon cytospin 3) was run at a setting of 700rpm for 5 minutes with high acceleration.
6. The cytospin preparation was then evaluated for cell density. If the cell population was too dense for analysis then the sample was further diluted and the procedure repeated until a slide of adequate cellular distribution was available for analysis.
7. The prepared BALF slide was stained using modified wrights stain. The slide was labeled and a cover slip placed in order to permanently fix the slide.
8. Each slide had the label covered by a number in order to blind the reviewer as to the identification of the slide to avoid bias.
9. The slide was placed on a light microscope and 200 cells counted and the differential cell count recorded.
10. All differential cell counts were performed by the primary investigator using a hemocytometer and these values used for statistical analysis.
11. Two separate investigators performed differential cell counts on 20 slides each to ensure the accuracy of the recordings.

E. Cytokine assessment: RNA isolation using QIAGEN RNeasy mini kit

1. All surfaces were cleaned with an RNase inhibitor and gloves were worn at all times during the procedure.
2. The cells were thawed at room temperature.
3. 70% ethanol was added to the cells depending on the number of cells present. This was then mixed well.
4. 700µl of the sample was then placed into a RNeasy mini column placed in a 2ml collection tube. The collection tube was then closed and centrifuged at 10,000rpm for 15 seconds. The flow-through was then discarded.
5. 350µl of buffer RW1 was then pipette into the RNeasy mini column and centrifuged for 15 seconds at 10,000rpm. The flow through was then discarded.
6. DNase 1 stock solution (12µl) was added to 70µl of buffer RDD and mixed carefully.
7. The DNase 1 incubation mix was then placed onto an RNeasy silica-gel membrane and left on the bench top for 30 min.
8. 350µl of buffer RW1 was then placed onto the RNeasy mini column and centrifuged for 15 seconds at 10,000rpm. The flow through was disgarded.
9. The RNeasy column was then transferred to a new 2 ml collection tube. 500µl of the buffer RPE was pipette into the RNeasy column. This was centrifuged for 15 seconds at 10,000rpm and the flow through was discarded.
10. Another 500µl of the buffer RPE was added to the RNeasy column and centrifuged for 2 minutes at 10,000rpm to dry the RNeasy silica-gel membrane.

11. The RNeasy column was then carefully removed and transferred to a new collection tube. To elute the RNA, 50µl of RNase free water is applied directly to the silica-gel membrane.
12. The resultant RNA was reverse transcribed and the resultant cDNA concentration measured using spectrophotometry.

F. RT- PCR using BIO_RAD iQ SYBR Green Supermix

BALF cells were selected for analysis of gene expression to compare expression of IL4 and IFNγ during the S1, S3 and S5 sample period of this study. These sample times were chosen as it was considered the best potential period to demonstrate a difference between and within the RAO-affected and control horse groups.

Reaction Set Up

Component concentration	**Volume per reaction**	**Final**
iQ SYBR Green Supermix	12.5 µl	1X
Sense Primer	0.25 µl	300nM
Anti-sense Primer	0.25 µl	300nM
Nuclease Free Water	11 µl	
DNA template	1 µl	
Total Volume	25µl	

1. Primers:

Primers were used at a concentration of 30 mM .This yields a 300 nM final concentration. cDNA amplification was achieved using the following primers:

CYTOKINE	**SENSE PRIMER**	**Tm(◦C)**	**ANTISENSE PRIMER**	**Tm(◦C)**
IL-4	AATGCCTGAGCGGACTG	55.5	TGCTCTTCTTGGCTTCATTC	54.1
INF-γ	TGAAGGTCCAGCGCAAAGC	59.5	CTGACTCCTCTTCCGCTTCC	57.9

2. PCR Protocol:

a. iQ SYBR Green Supermix (Invitrogen; Carlsbad, CA), cDNA(diluted 1:10) – RT, and the primers for IL-4 and IFNγ were thawed on the bench top at room temp. All components were then lightly vortexed before use.

b. 24 µl of sample was then dispensed into 1.65ml microcentrifuge tubes in triplicate.
c. Add 1µl of cDNA or –RT template to tube. Total volume was 25 µl. For each cDNA sample triplicate reactions were performed on each plate. Also included on each plate were a positive and a negative control. The plates were set up with all the standards for one cytokine contained in each plate.

d. 25µl samples were pipetted into 96 well RT-PCR plate using a new RNase-free filter pipette tip for each well.

e. Each plate was covered with a heat sealing optically clear tape and heat sealed for about 5 seconds.

f. The whole plate was then centrifuged for 1 minute at 400 x g at room temp.

g. The plate was then loaded into the BIO RAD iCycler170-8740 iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and the program set according to our specific protocol and necessary annealing temperatures. Specific annealing temperatures were set for each cytokine based on the optimal annealing temperature determined by the temperature gradient analysis protocol. Each PCR reaction consisted of a 3 step melt protocol as follow: 95 °C for 3 minutes; 40 cycles of 10 seconds at 95 °C followed by 15 seconds at 55 °C (annealing temperature) followed by 20 seconds at 72 °C (extension step); then 1 minute at 95 °C, followed by 1 minute at 55 °C; then 80 cycles were run at 55 °C for 10 seconds each to determine the melt curve. The amount of fluorescence was measured for each sample during the PCR reaction. The amount of mRNA expression for each cytokine was obtained from the different standards dilutions.

h. The endpoint, C_T, is the cycle number corresponding to the detection of product formation. Data was log transformed (ln) and expressed as a ratio of start quantity of IL4 to start quantity of IFNγ.

i. The ratio of IL-4 to IFNγ was then calculated. The validation of primers and probes for equine IFN-γ and IL-4 has been previously reported (Ainsworth et al., 2003)

Figures

Figure 1: Response to particle inhalation

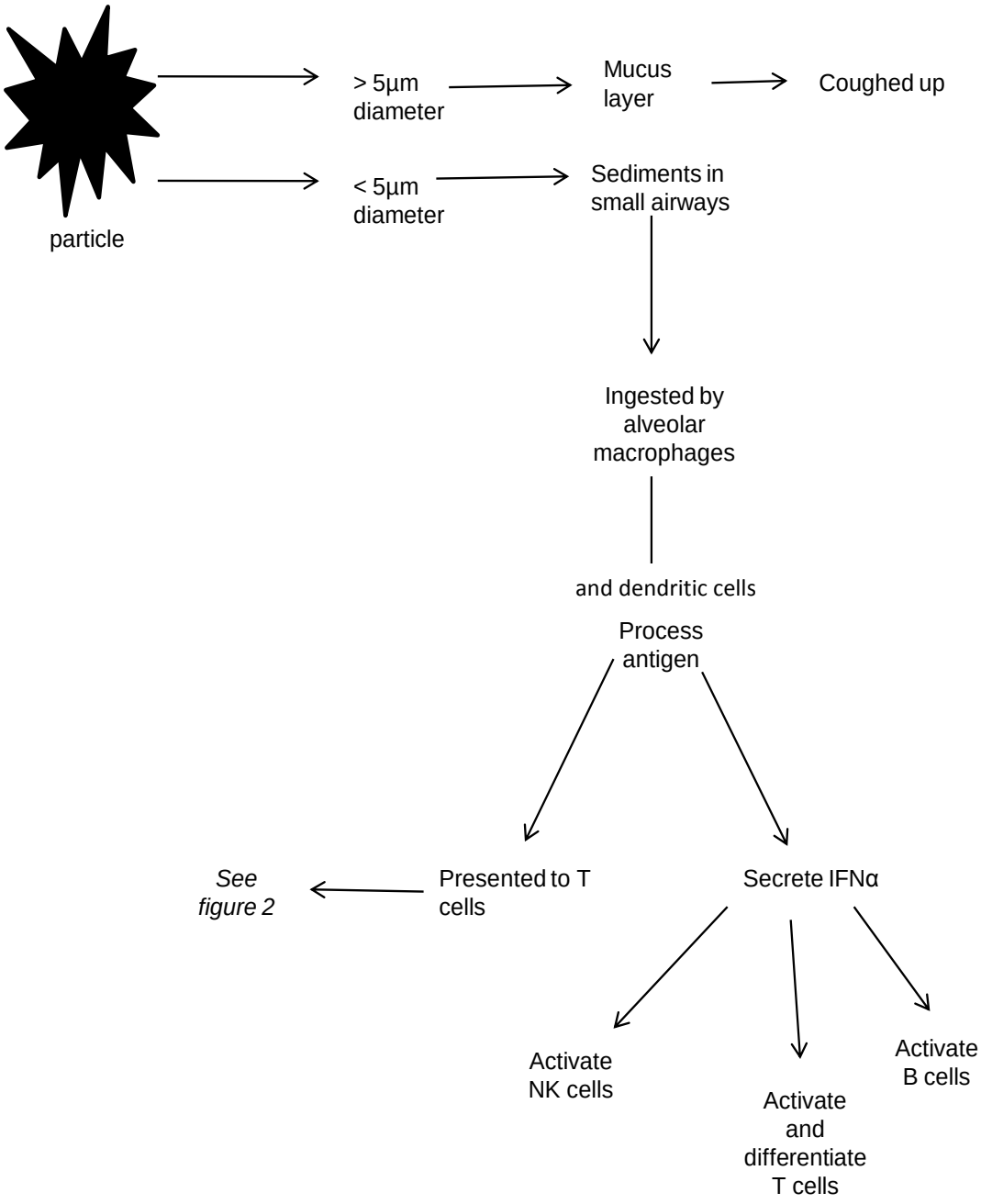


Figure 2: T cell activation

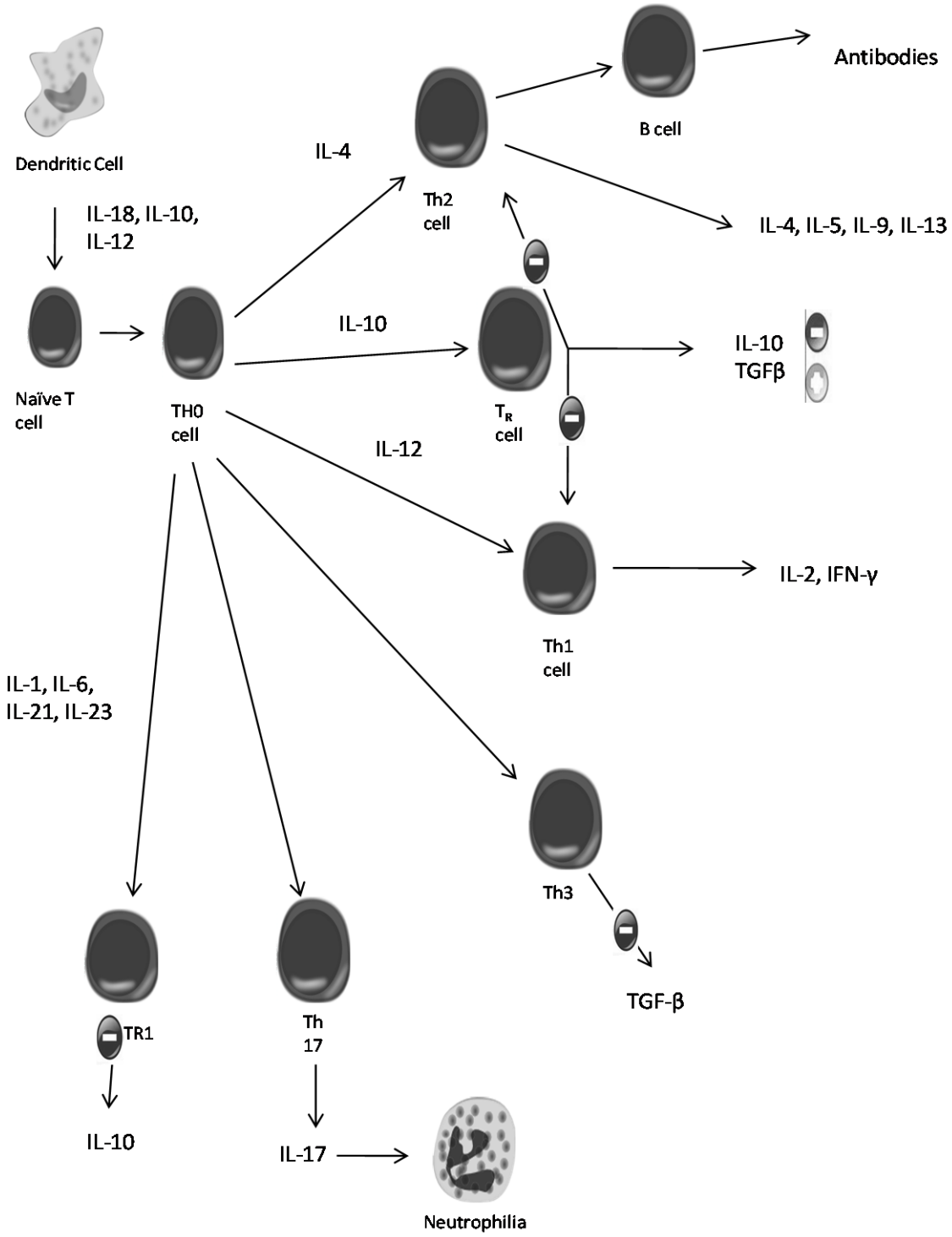


Figure 3: Processing to determine cytokine expression

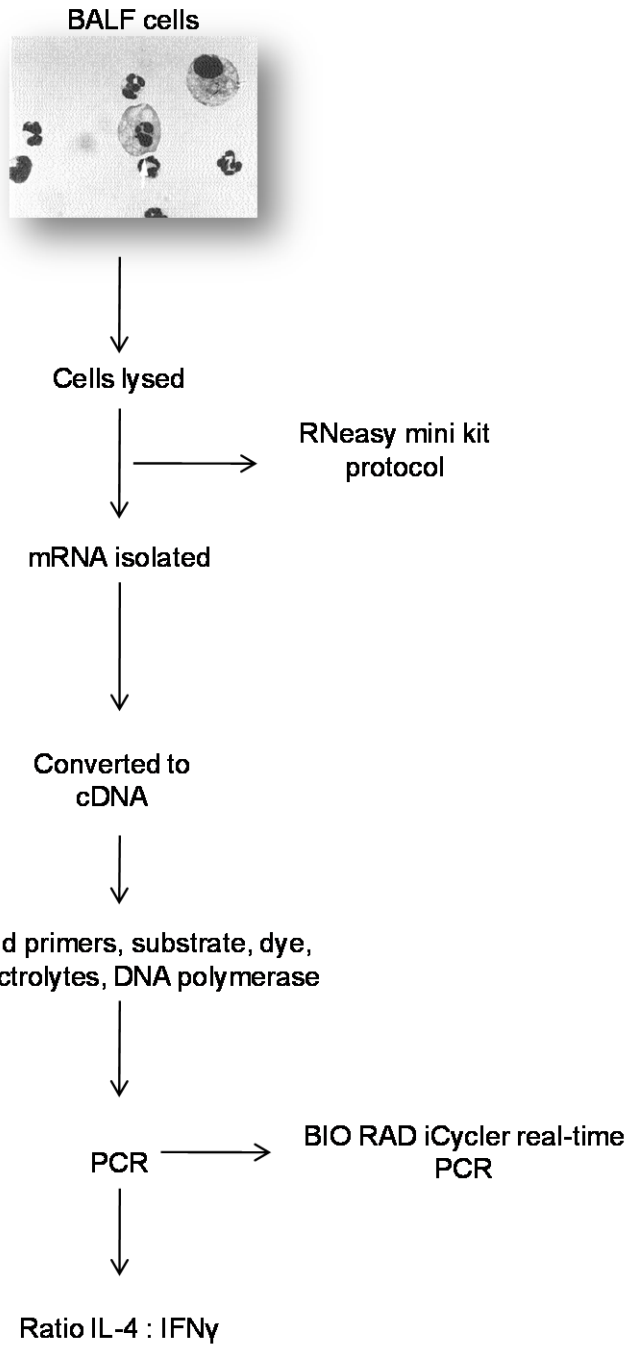


Figure 4-9:

Graphs are of mean data of 6 horses receiving one of three treatments for RAO.
Treatments included **DEX:** tapering dosage regime of intravenous dexamethasone treatment without environmental modification; **DEX/ENV**: tapering dosage regime of intravenous dexamethasone treatment with environmental modification; and **ENV**: environmental modification with an intravenous saline placebo.
Samples were taken at day 0 (Pretreatment), 3, 6, 12 and 21 of treatment.

Key to designation of statistical significance for Figures 4-9

a: Value differs significantly from pretreatment (sample 1) value within the same treatment group

b: Indicates that the values differed between two subsequent Sample times within the same Treatment group. The two sample points that differ are indicated by the letter "b" followed by a common number. For example, if in Treatment group 1, if there was a significantly different between Sample 3 and Sample 4, then each value will have "b1" next to the value for that Sample time.

c: Values collected at a Sample period differ significantly between two Treatment groups. Values that differ will be signified by the letter "c" followed by a number

Figure 4: Clinical Scores. The comparison between the three treatments demonstrating the mean abdominal push scores (figure 4a), mean nasal flare (figure 4b) scores and the mean total clinical scores (Figure 4c) over the 21 day treatment period.

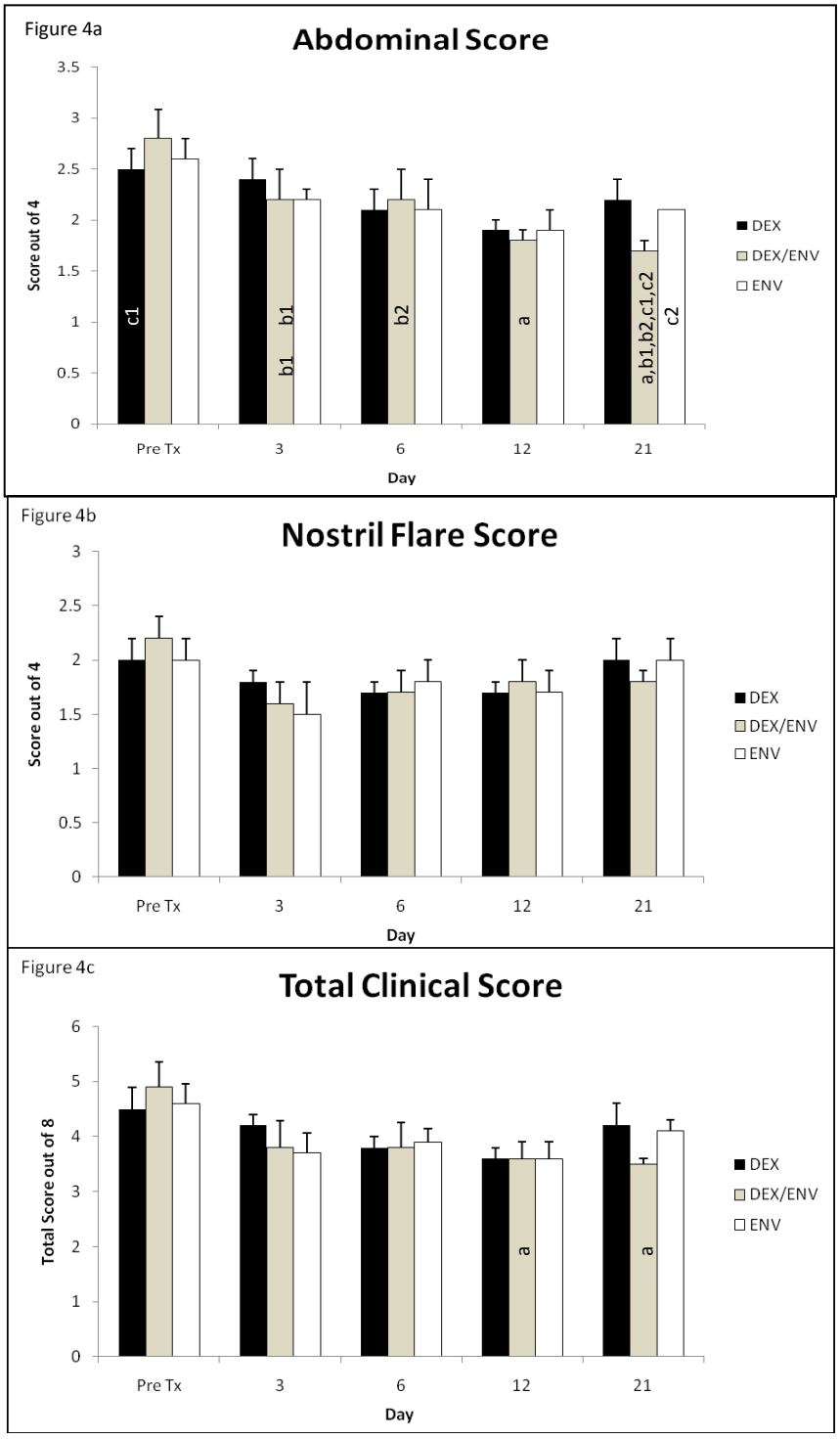
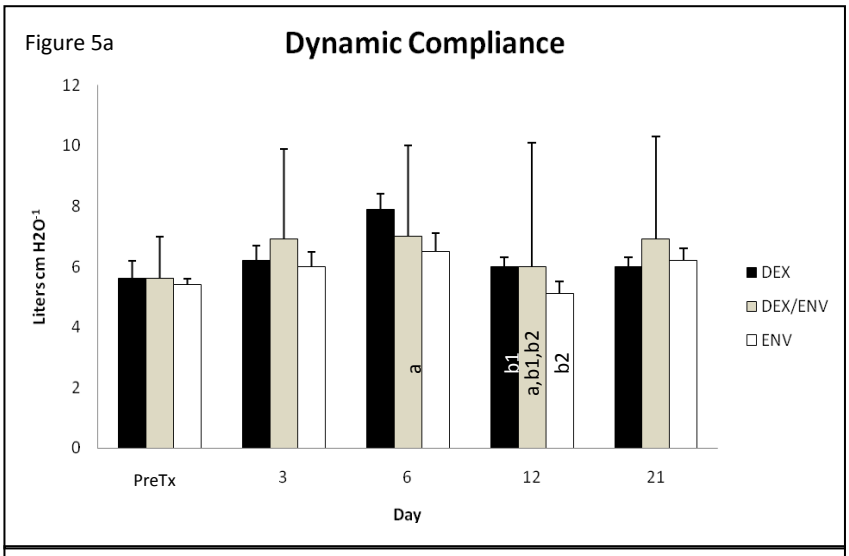
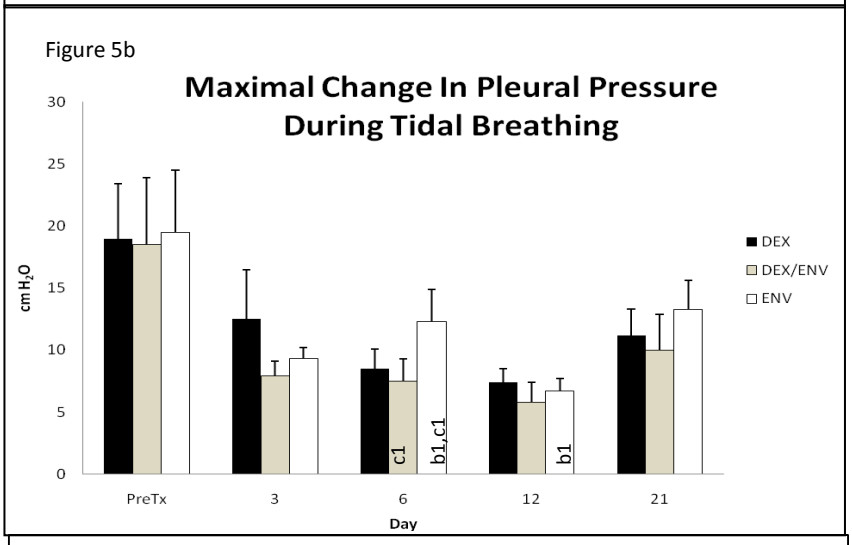


Figure 5: Pulmonary lung function between the three treatments demonstrating changes in the dynamic compliance (figure 5a: C_{dyn} in liters per cm H_2O), maximum change in pleural pressures during tidal breathing (figure 5b: ΔPpL_{max} in cmH_2O), and tidal volume (figure 5c: TV in liters) over the 21 day treatment period.





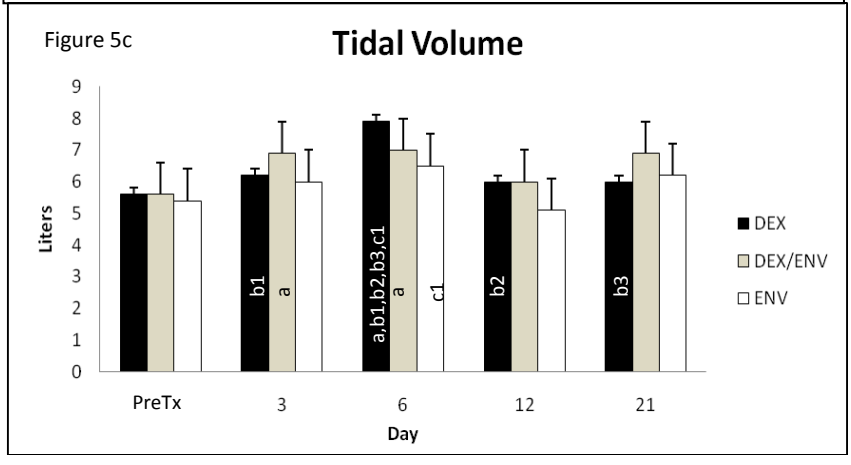


Figure 6: The comparison between the mean volume of BALF (ml) (figure 6a) and total cells x 10^7 (figure 6b) retrieved from each treatment group at each sample time over a 21 day treatment period.

Figure 6a:

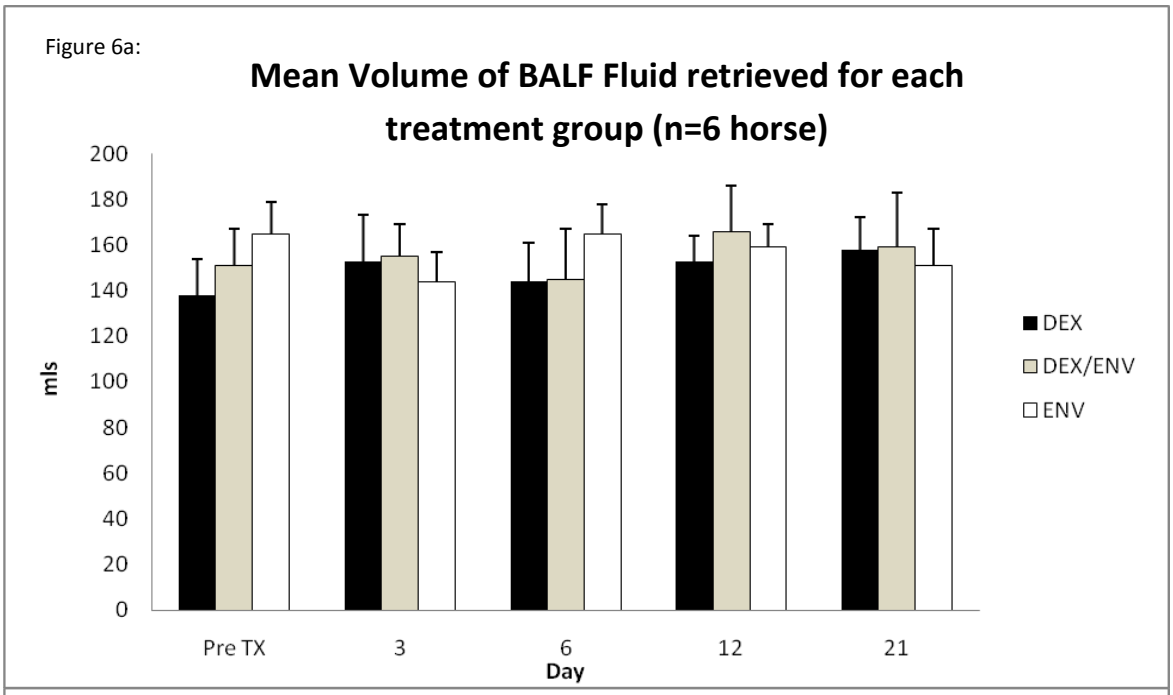


Figure 6b:

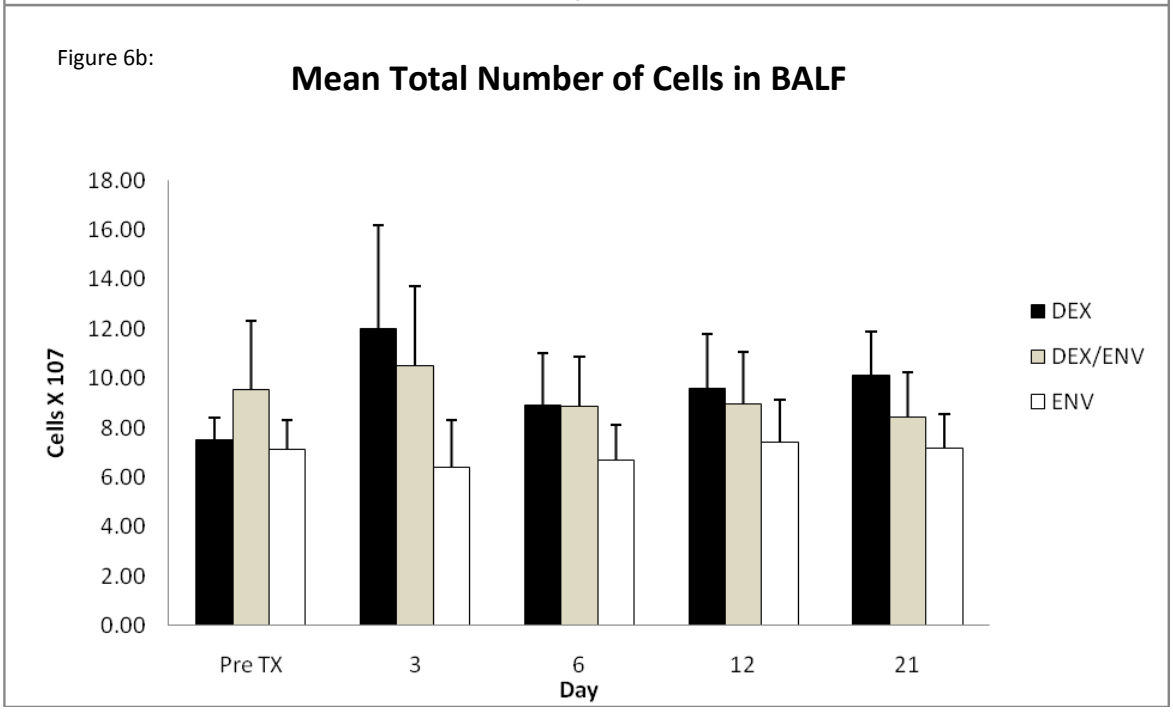


Figure 7: Bronchoalveolar lavage fluid (BALF) cytology. The comparison between the three treatments over the 21 day period demonstrating the percentage (%) of lymphocytes (figure 7a) and total number of lymphocytes x 10^6 (figure 7b) in the BALF.

Figure 7a

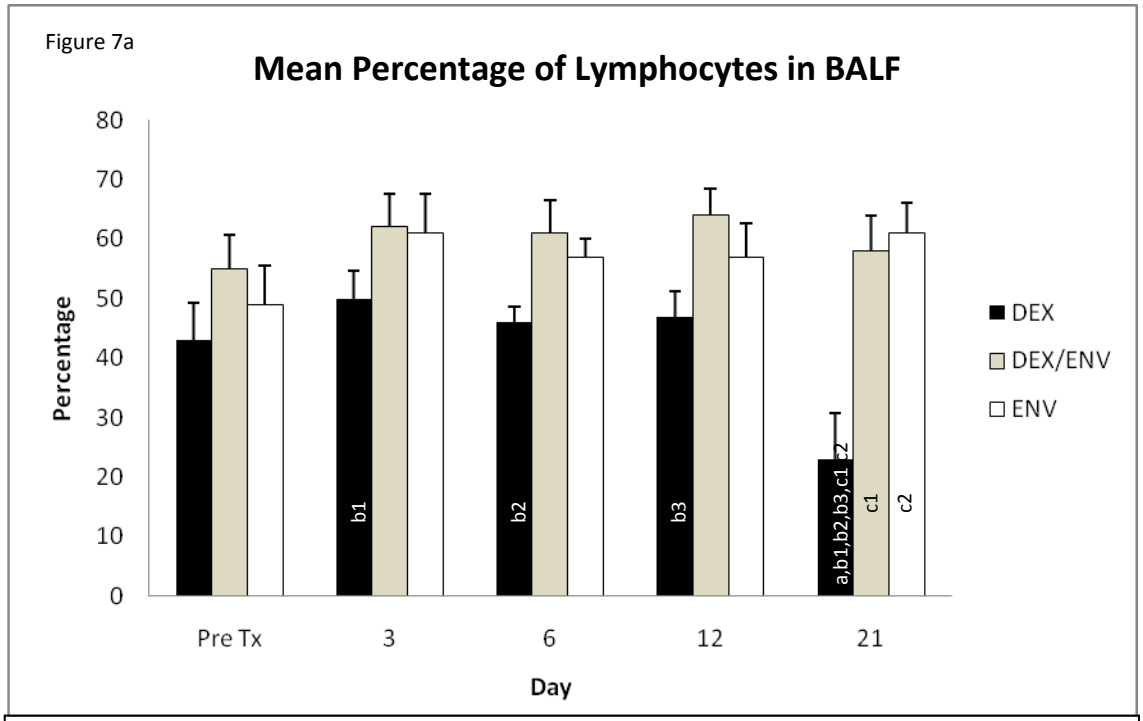


Figure 7b

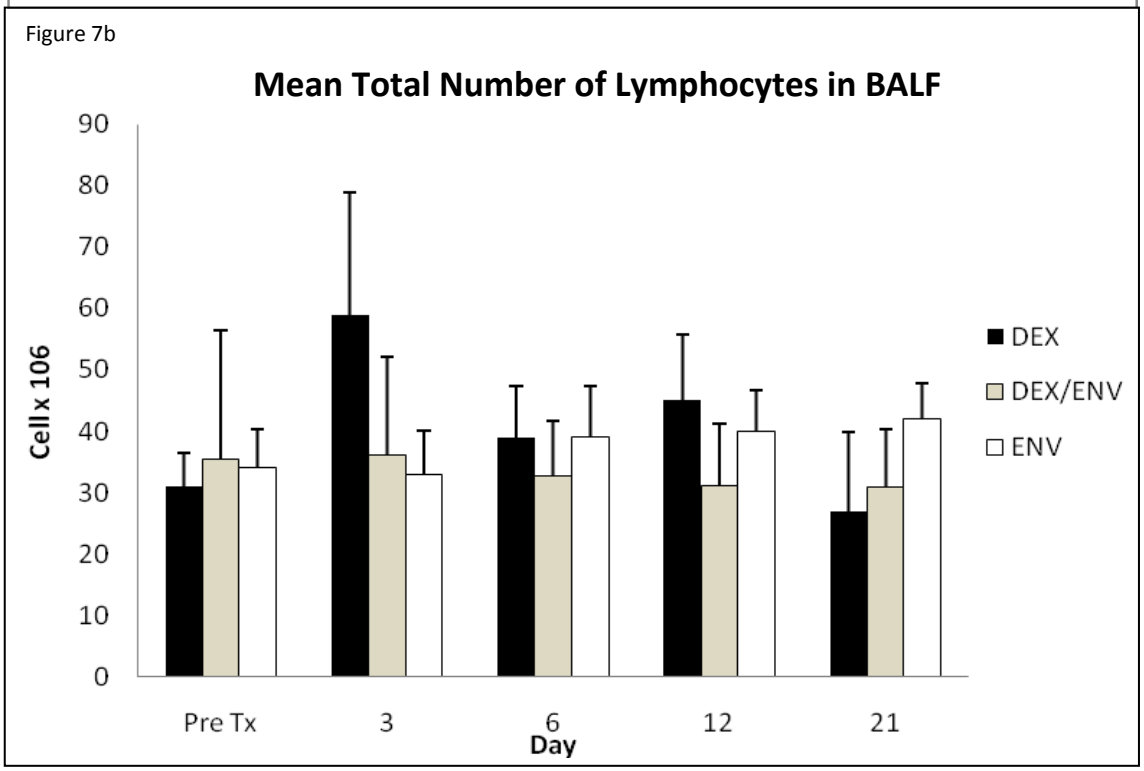


Figure 8: Bronchoalveolar lavage fluid (BALF) cytology. The comparison between the three treatments over the 21 day period demonstrating the percentage (%) of macrophages (figure 8a) and total number x 10^6 of macrophages (figure 8b) in the BALF.

Figure 8a

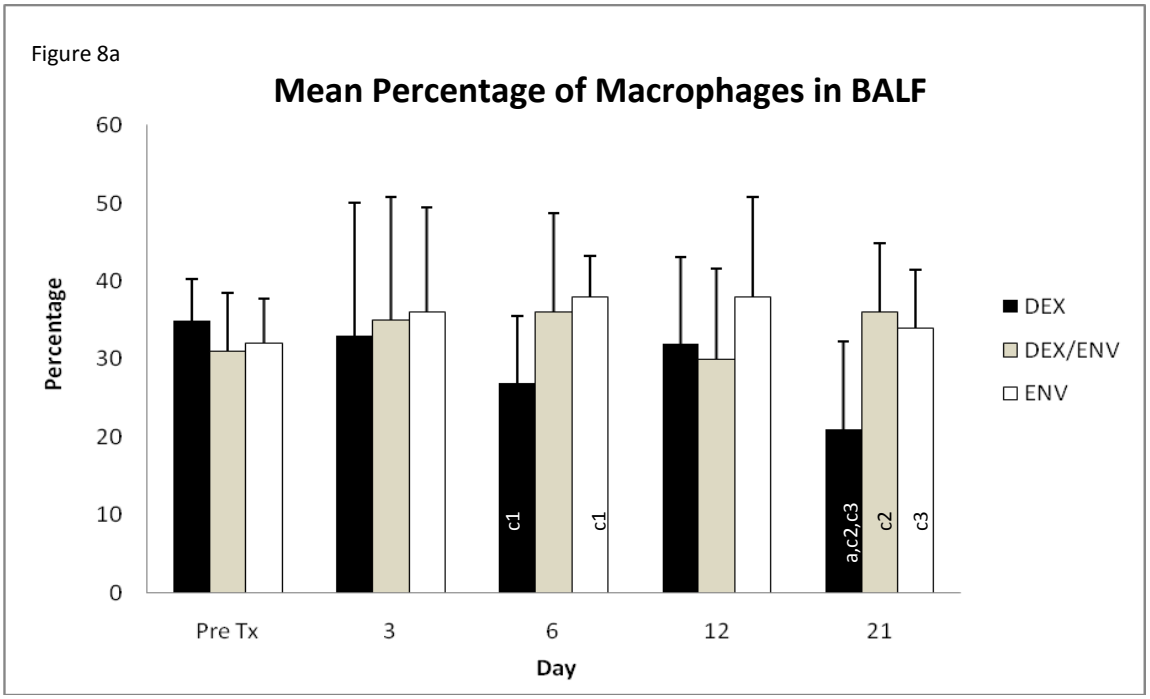


Figure 8b

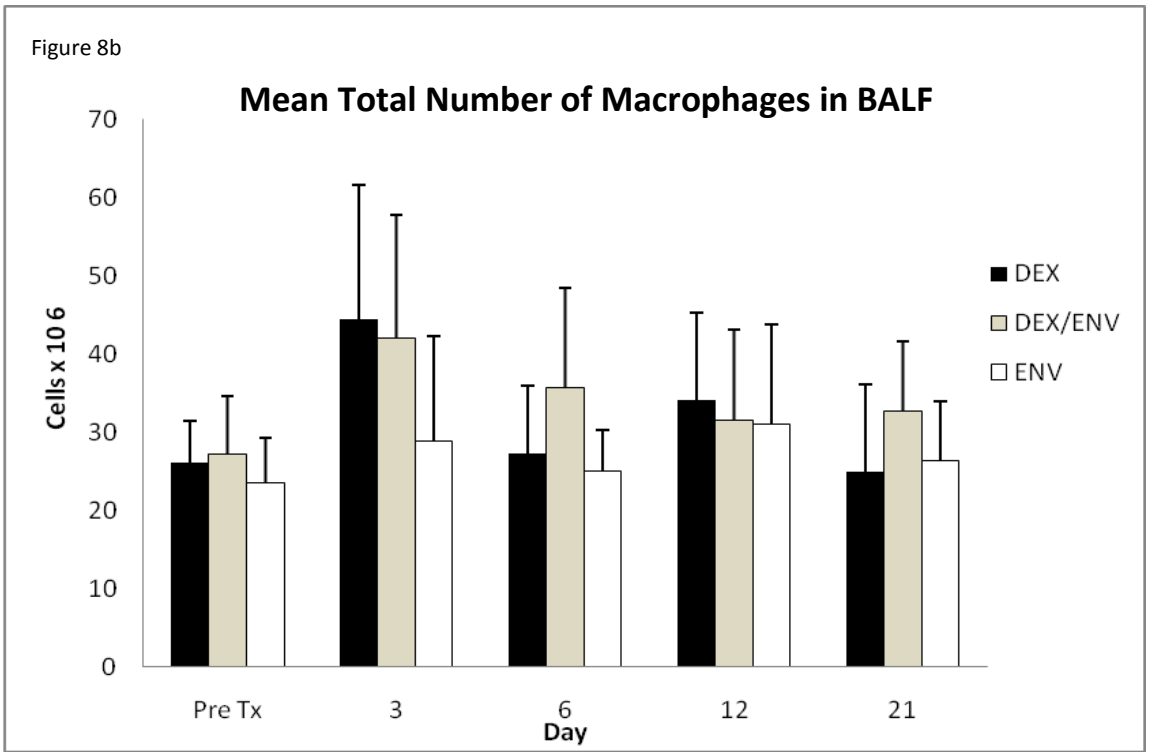


Figure 9: Bronchoalveolar lavage fluid (BALF) cytology. The comparison between the three treatments over the 21 day period demonstrating the percentage (%) of neutrophils (figure 9a) and total number x 10^6 of neutrophils (figure 9b) in the BALF.

Figure 9a

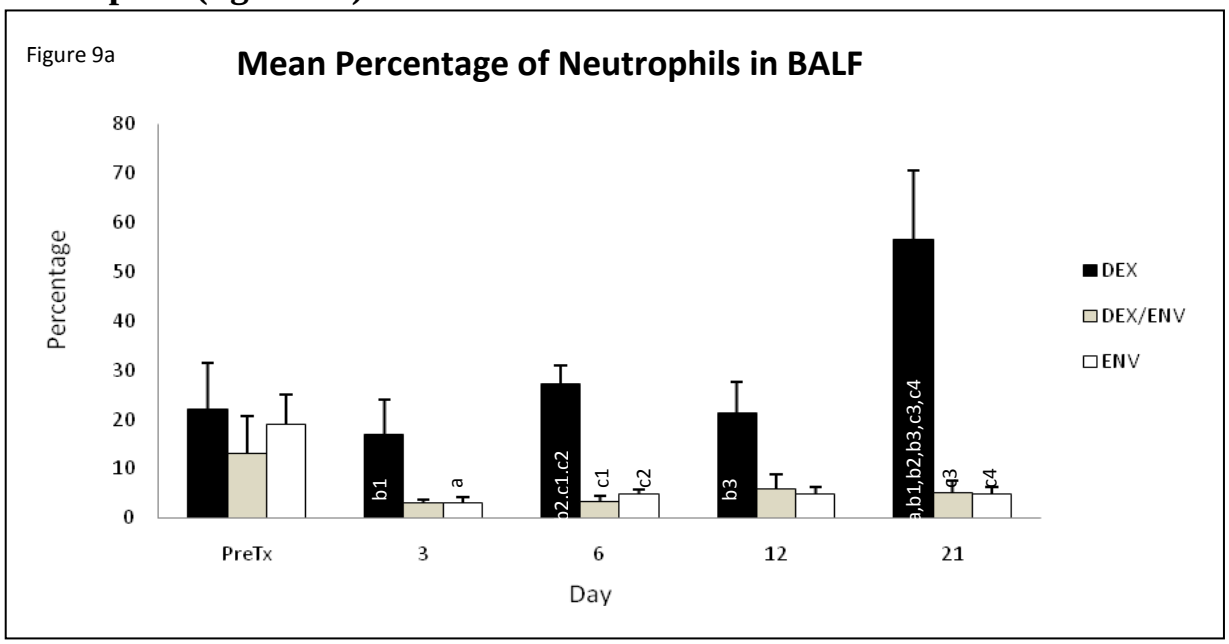


Figure 9b

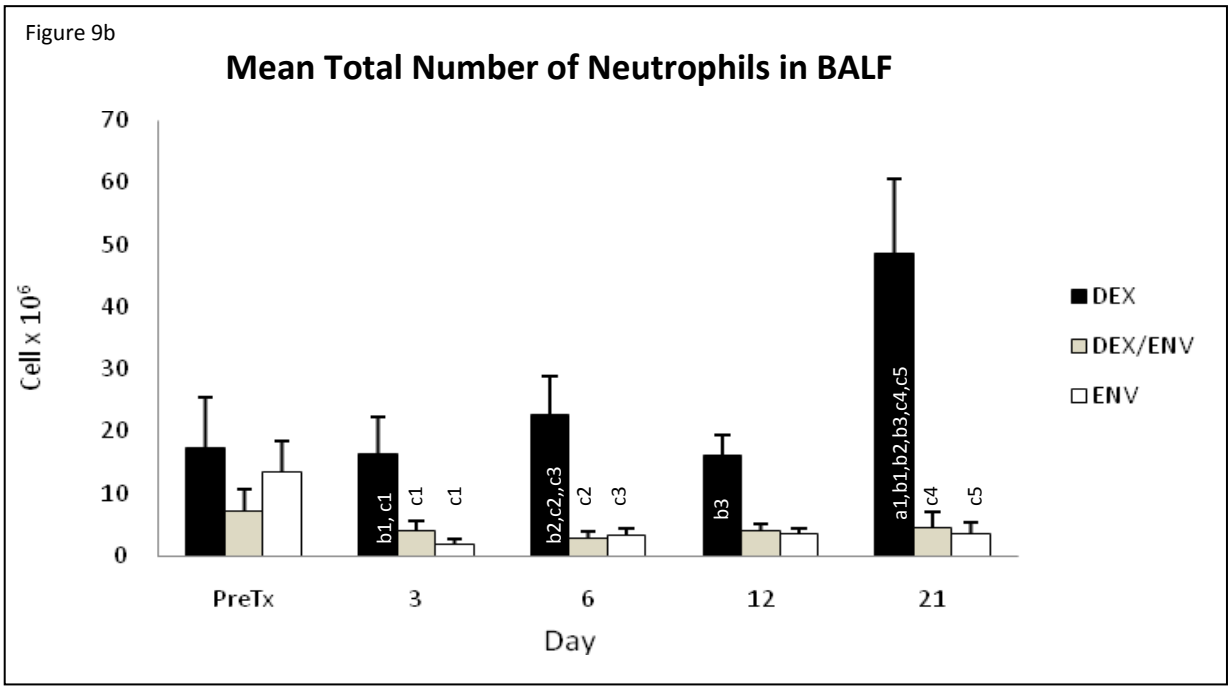


Figure 10: BALF neutrophil percentages taken from the six individual horses in the DEX treatment group over the 21 day treatment period.

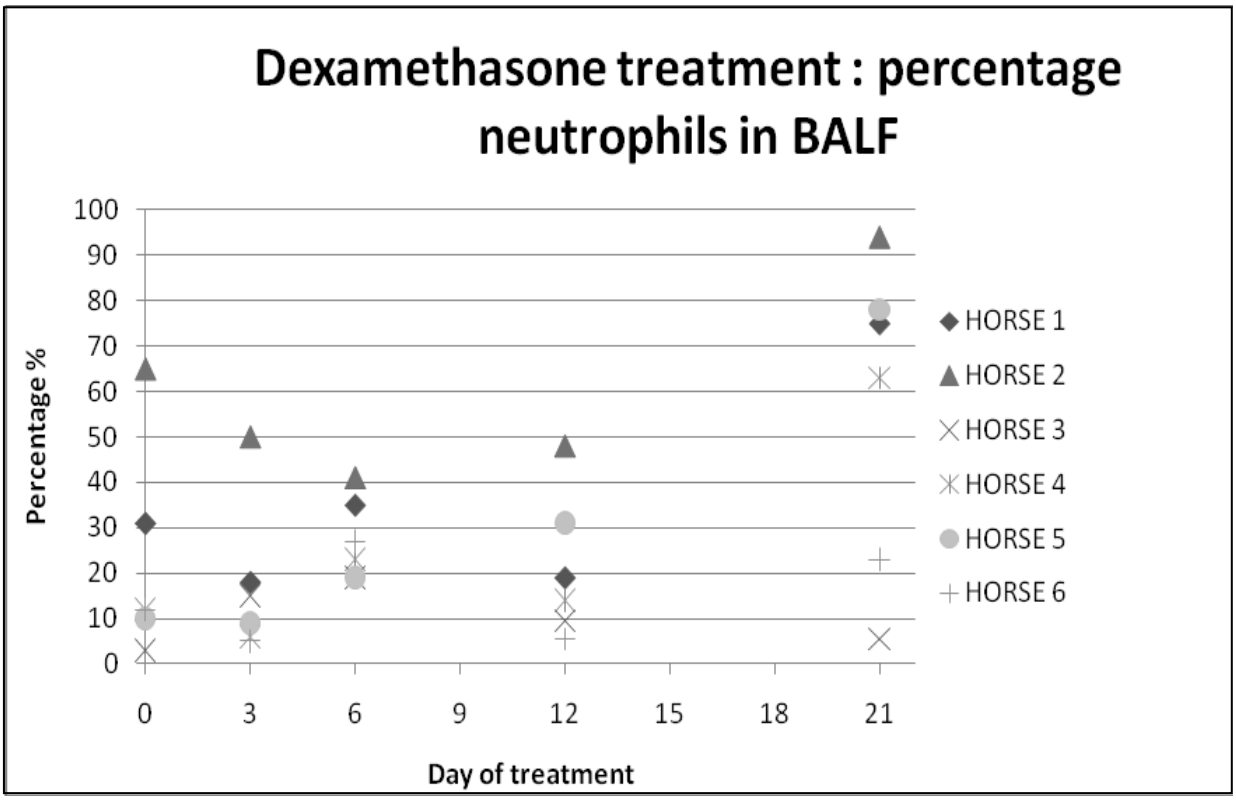


Figure 11: Overlay of the total number of neutrophils (cells x 1,000,000) in the BALF of horses in the dexamethasone treatment group (DEX) vs. the total clinical score (TCS).

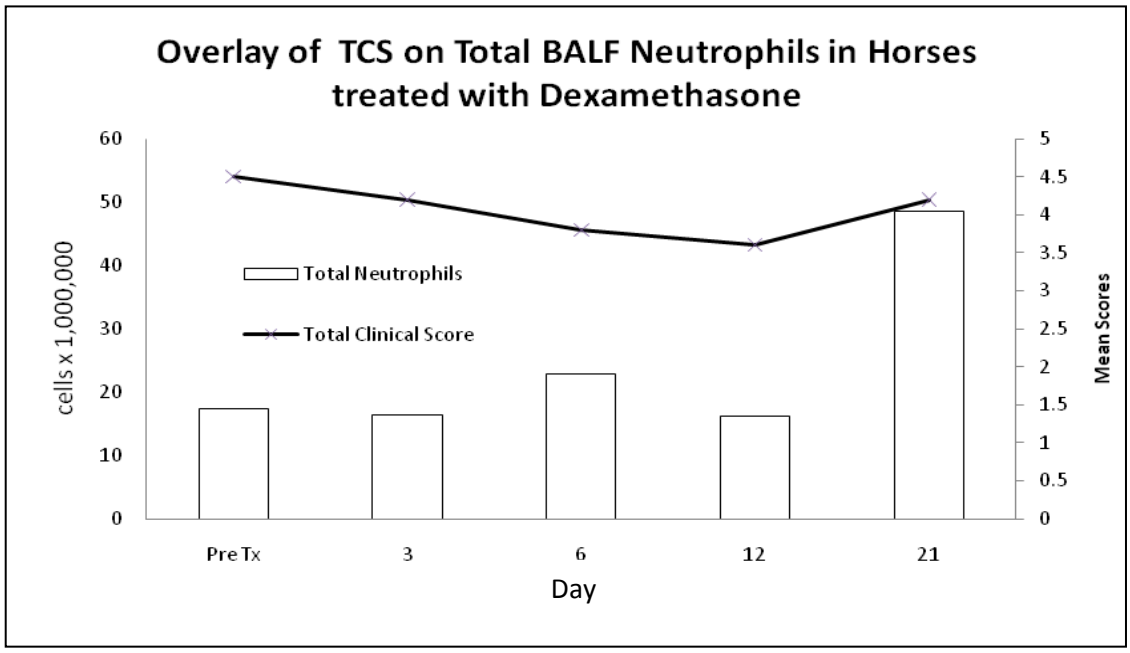


Figure 12: Overlay of the total number of neutrophils (cells x 1,000,000) in the BALF of horses in the dexamethasone treatment group (DEX) vs. the maximum change in pleural pressures (cm H_2O).

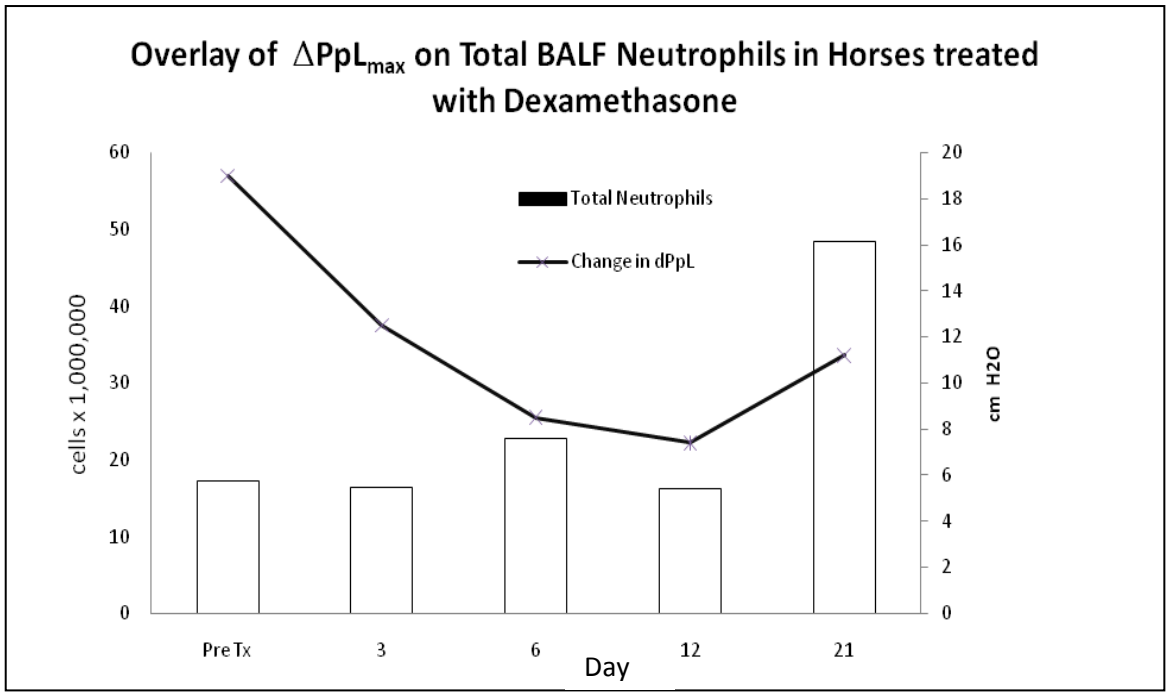


Figure 13: Overlay of Total Neutrophils (x 10^6) and ratio of IL4/INFγ in the group treated with dexamethasone (figure 13a), dexamethasone and environmental changes (figure 13b) and environmental changes (figure 13c) over the 21 day treatment period.

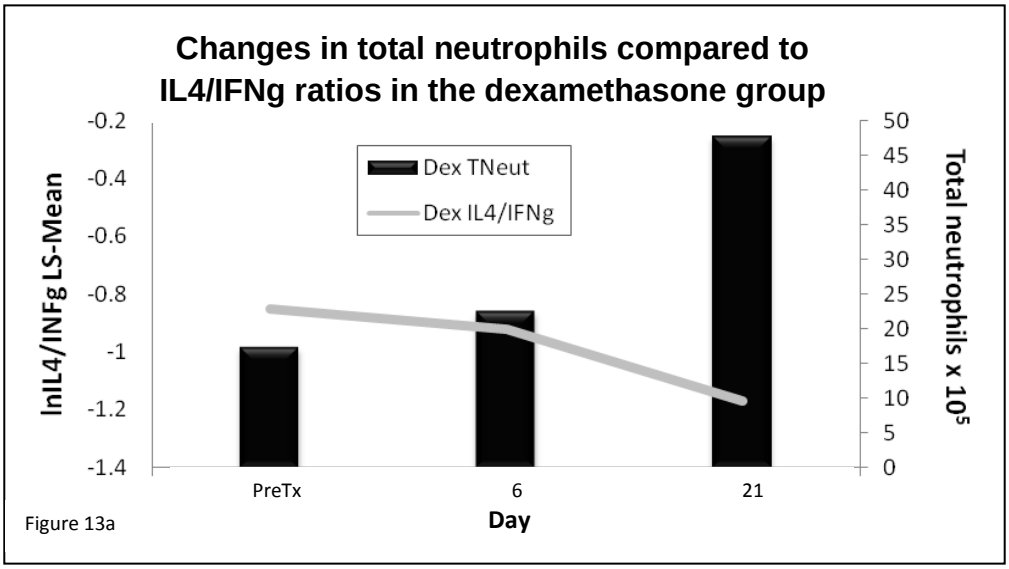


Figure 13a

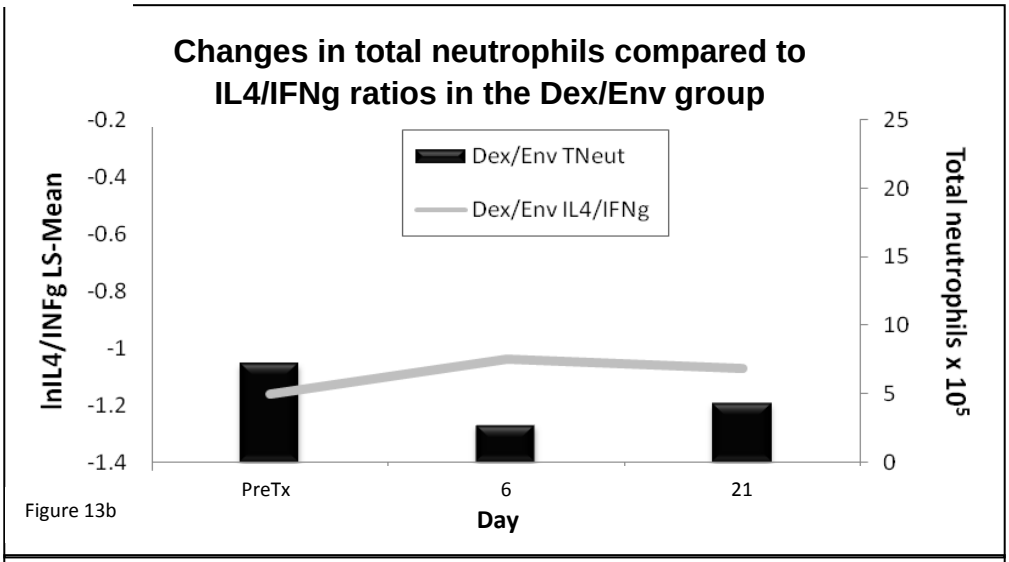


Figure 13b

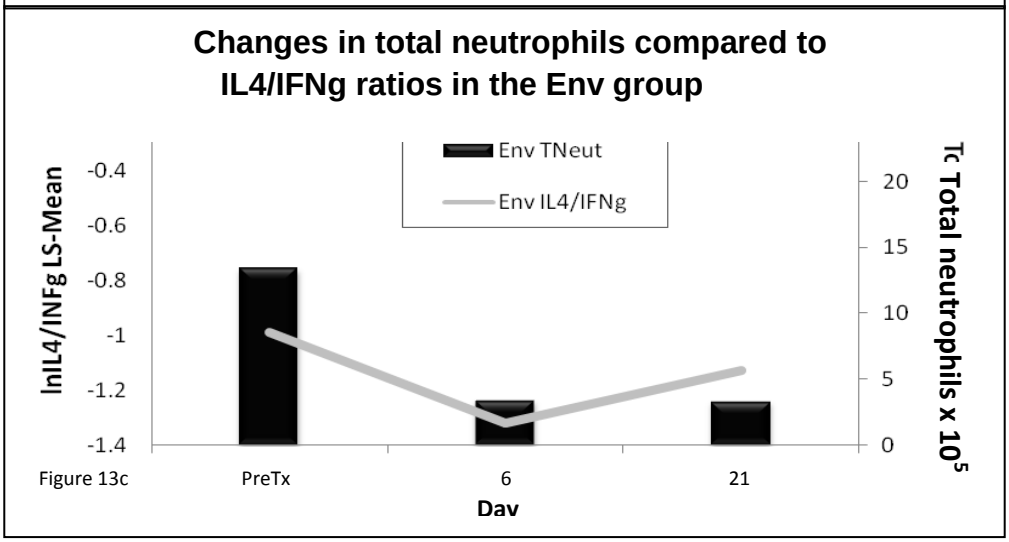


Figure 13c

Tables

Table 1: Clinical score

Adapted from Rush et al. Cytologic evaluation of bronchoalveolar lavage fluid from horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **1998**;59:1033-1038.

Nasal flare (NF)	Score	Abdominal push (AP)
No flaring	1	No abdominal component to breathing
Slight, occasional flaring of the nostrils	2	Slight abdominal component to breathing
Moderate nostril flaring	3	Moderate abdominal movement
Severe continuous flaring during each respiration	4	Severe, marked abdominal movement

Added to give total clinical score

Total clinical score	Clinical signs
2	No signs
3	Mild signs
4	Mild signs
5	Moderate signs
6	Moderate signs
7	Severe signs
8	Severe signs

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Sample (S)*	S1			S2			S3						S4									S5
Treatment (T)	T	T	T	T	T	T	T		T		T		T			T			T			
Dexamethasone dose IV	0.1mg/kg q 24 h			0.05mg/kg q 24 h			0.05 mg/kg q 48 h						0.05 mg/kg q 72 h									

*Sample always collected prior to the administration of treatment

Table 2: Treatment protocol in relation to sample collection periods (S1 – S5)

Table 3: Mean of Clinical Parameters and Scores at each sample time from horses in the three treatment groups

Sample	Treatment	HR	(range)	RR	(range)	TEMP	(range)	APS	(range)	NFS	(range)	TCS	(range)
1	DEX	42	(36-48)	21	(12-36)	99.0 [c1]	(98.8-99.4)	2.5	(2-3)	2	(1.5-3)	4.5	(3.5-6)
2	DEX	40	(32-48)	18	(12-36)	99.2	(98.3-99.6)	2.4	(2-3)	1.8	(1.5-2)	4.2	(3.5-5.0)
3	DEX	38 [b1]	(36-40)	15	(12-27)	99.6	(98.9-100.3)	2.1	(1.5-2.5)	1.7	(1.5-2)	3.8	(3-4.5)
4	DEX	35 [a,b1, b2]	(32-36)	15	(12-16)	99.0	(98.3-99.4)	1.9	(1.5-2)	1.7	(1.5-2)	3.6	(3-4)
5	DEX	41 [b2]	(36-52)	17 [c1]	(12-20)	99.3	(98.8-99.8)	2.2 [c1]	(1.5-2.5)	2	(1.5-2.5)	4.2	(3-5)
1	DEX/ENV	38	(36-40)	19	(8-28)	99.6 [c1, c2]	(99.1-100.1)	2.8	(2-4)	2.2	(1.5-3)	4.9	(4-7)
2	DEX/ENV	40	(36-48)	15	(12-20)	99.4	(98.5-100.4)	2.2 [b1]	(1.5-3)	1.6	(1-2.5)	3.8	(2.5-5.5)
3	DEX/ENV	41 [c1, b3]	(40-44)	15	(12-20)	99.3	(97.3-100)	2.2 [b2]	(1-3)	1.7	(1-2.5)	3.8	(2.0-5.5)
4	DEX/ENV	35 [b3]	(32-42)	13	(12-20)	98.9 [a]	(98.5-99.2)	1.8 [a]	(1.5-2)	1.8	(1-2.5)	3.6 [a]	(2.5-4.5)
5	DEX/ENV	38	(32-40)	14	(12-16)	99.3	(99.8.-99.7)	1.7 [a,b1,b2,c1,c2]	(1.5-3)	1.8	(1.5-2)	3.5 [a]	(3-4)
1	ENV	40	(32-46)	18	(12-24)	99.1 [c2]	(98.3-99.6)	2.6	(2-3)	2	(1.5-3)	4.6	(3.5-6)
2	ENV	37	(32-44)	13	(13-30)	99.3	(98.8-99.7)	2.2	(2-2.5)	1.5	(1.0-2.5)	3.7	(3-5)
3	ENV	37 [c1]	(32-40)	12	(10-16)	99.3	(98.8-99.6)	2.1	(1.5-3)	1.8	(1.5-2.5)	3.9	(3-4.5)
4	ENV	34 [a]	(32-36)	13	(12-16)	98.9	(98.2-99.8)	1.9	(1.5-2.5)	1.7	(1-2)	3.6	(2.5-4.5)
5	ENV	36	(34-40)	13 [c1]	(8-16)	98.8	(98.0-99.6)	2.1 [c2]	(2-2.5)	2	(1.5-2.5)	4.1	(3.5-4.5)

For all measurements n=6 except Treatment DEX/ENV Sample Period Number 4 and 5 as one horse was withdrawn from the study for these periods. For those sample sets, n=5.

Treatments:

DEX: Descending doses and frequency of administration of IV dexamethasone as described in methods section
DEX/ENV: Descending doses of IV dexamethasone coupled with a change in environment.
ENV: Change in environment with IV injections of saline of equal volume and frequency as the appropriate dose of dexamethasone

HR: The mean heart rate, expressed as heart beats per minute
RR: The mean respiratory rate, expressed as breaths per minute
TEMP: The mean temperature in degrees Fahrenheit
APS: The mean abdominal push score, based on a previously described scale of 1-4 with, 1 being normal and 4 being obvious abdominal effort throughout the respiratory cycle
NFS: The mean nostril flare score, based on a previously described scale of 1-4 with, 1 being normal and 4 being nostrils fixed in a flare
TCS: The total clinical score which is the sum of APS and NFS

a: Value differs significantly from pretreatment (sample 1) value within the same treatment group
b: Indicates that the values differed between two subsequent Sample times within the same Treatment group. The two sample points that differ are indicated by the letter ”b” followed by a common number. For example, if in Treatment group 1, if there was a significantly different between Sample 3 and Sample 4, then each value will have “b1” next to the value for that Sample time.
c: Values collected at a Sample period differ significantly between two Treatment groups. Values that differ will be signified by the letter “c” followed by a number

Table 4: Mean parameters of Pulmonary Function at each sample time from horses in the three treatment groups

Sample	Treatment	ΔPpL_{max}	(SEM)	C_{dyn}	(SEM)	TV	(SEM)	T_i	(SEM)	T_e	(SEM)	R_L	(SEM)
1	DEX	19.0	4.4	2.1	0.6	5.6	0.6	1.9	0.2	3.5	0.6	1.6	0.6
2	DEX	12.5	4.0	2.0	0.5	6.2 [b1]	0.9	2.1	0.3	3.4	0.5	1.2	0.5
3	DEX	8.5	1.6	2.9	0.5	7.9 [a,b1,b2,b3,c1]	0.9	1.9	0.3	3.0	0.7	0.6 [c1]	0.2
4	DEX	7.4	1.1	1.8 [b1]	0.3	6 [b2]	0.4	2.5	0.3	3.4	0.4	0.9	0.2
5	DEX	11.2	2.1	1.5	0.3	6 [b3]	0.5	2.0	0.1	2.8 [c1,c2]	0.2	0.9	0.2
1	DEX/ENV	18.5	5.4	1.4	0.3	5.6	0.4	1.7	0.3	3.0	0.6	1.2	0.2
2	DEX/ENV	7.9	1.2	3.0	0.9	6.9 [a]	0.5	2.7 [a]	0.3	4.0	0.5	0.8	0.3
3	DEX/ENV	7.5 [c1]	1.8	3.0 [a]	0.7	7 [a]	0.4	2.5	0.4	3.2	0.4	0.8	0.3
4	DEX/ENV	5.8	1.6	4.1 [a,b1, b2]	1.4	6	0.5	2.7 [a]	0.3	3.7	0.4	0.6	0.2
5	DEX/ENV	10.0	2.9	3.4	1.6	6.9	0.3	2.6 [a]	0.4	3.8[c1]	0.6	0.7	0.2
1	ENV	19.5	5.0	1.4	0.2	5.4	0.6	1.8	0.3	3.5	0.8	1.8	0.7
2	ENV	9.3	0.9	2.2	0.5	6	0.6	2.7 [a]	0.3	4.0	0.5	0.9	0.2
3	ENV	12.3 [b1,c1]	2.6	2.1	0.6	6.5 [c1]	0.4	2.4	0.2	3.4	0.4	0.9 [c1]	0.2
4	ENV	6.7[b1]	1.0	1.8 [b2]	0.4	5.1	0.5	2.5	0.3	3.7	0.6	1.1	0.3
5	ENV	13.3	2.3	1.8	0.4	6.2	0.9	2.2	0.4	3.8 [c2]	0.7	1.3	0.4

For all measurements n=6 except DEX/ENV Sample Period Number 4 and 5 as one horse was withdrawn from the study for these periods. For those sample sets, n=5. Pressure changes were recorded manually. All other parameters were measured using the Buxco System, which is described in the methods section.

Treatments:
DEX: Descending doses and frequency of administration of IV dexamethasone as described in methods section
DEX/ENV: Descending doses of IV dexamethasone coupled with a change in environment.
ENV: Change in environment with IV injections of saline of equal volume and frequency as the appropriate dose of dexamethasone
ΔPpL_{max} = Maximal change in pleural pressure during tidal breathing, expressed as cm H_2O. Normal Range
C_{dyn} = Dynamic compliance, expressed as liters cm H_2O^{-1}
TV = Tidal volume in liters
Ti = Inspiratory time in seconds
Te = Expiratory time in seconds
R_L= Lung Resistance liters cm H_2O/L x second
See reference 16. (Derksen et.al 1985) for normal values

a: Value differs significantly from pretreatment (sample 1) value within the same treatment group
b: Indicates that the values differed between two subsequent Sample times within the same Treatment group. The two sample points that differ are indicated by the letter "b" followed by a common number. For example, if in Treatment group 1, if there was a significantly different between Sample 3 and Sample 4, then each value will have "b1" next to the value for that Sample time.
c: Values collected at a Sample period differ significantly between two Treatment groups. Values that differ will be signified by the letter "c" followed by a number

Table 5: Mean distribution of cells in BALF at each sample time from horses in the three treatment groups

Sample	Treatment	%Neut	SEM	TNeut	SEM	%Mac	SEM	TMac	SEM	%Lymph	SEM	TLymph	SEM
1	DEX	22	9.4	17.3	8.1	35	4	26.2	5.3	43	6.2	31	5.4
2	DEX	17 ^{b1}	6.9	16.4^{b1,c1}	5.9	33	5	44.5	17.1	50 ^{b1}	4.7	59	20
3	DEX	27 ^{b2,c1,c2}	3.7	22.8^{b2,c2,c3}	6	27^{c1}	4	27.3	8.6	46 ^{b2}	2.7	39	8.3
4	DEX	21 ^{b3}	6.5	16.2^{b3}	3.1	32	4	34.2	11.1	47 ^{b3}	4.3	45	10.8
5	DEX	56 $^{a,b1, b2, b3,c3,c4}$	14.1	48.5 ^{a,b1,b2,b3,c4,c5}	12.1	21^{a,c2,c3}	7	25.0	11.2	23 ^{a,b1,b2,b3,c1,c2}	7.7	27	12.9
1	DEX/ENV	13	7.6	7.2	3.4	31	6	27.2	7.5	55	5.7	60	21
2	DEX/ENV	3	0.6	4.0	1.5	35	5	42.2	15.8	62	5.5	58	16
3	DEX/ENV	3 ^{c1}	1.2	2.7^{c2}	1.2	36	6	35.7	12.7	61	5.5	50	9
4	DEX/ENV	6	2.8	4.0	1.1	30	6	31.6	11.6	64	4.5	54	10.1
5	DEX/ENV	5 ^{c3}	2.5	4.6^{c4}	2.4	36 ^{c2}	4	32.7	8.9	58^{c1}	5.9	46	9.3
1	ENV	19	6.1	13.4	5	32	3	23.6	5.7	49	6.5	34	6.4
2	ENV	3 ^{a}	1.1	1.8 ^{c1}	0.8	36	8	28.9	13.4	61	6.7	33	7.1
3	ENV	5 ^{c2}	1.0	3.4^{c3}	1	38 ^{c1}	3	25.0	5.3	57	3.1	39	8.3
4	ENV	5	1.3	3.5	1	38	6	31.0	12.8	57	5.6	40	6.7
5	ENV	5 ^{c4}	1.4	3.5^{c5}	1.9	34 ^{c3}	5	26.4	7.5	61^{c2}	5.1	42	5.9

For all measurements n=6 except DEX/ENV Sample Period Number 4 and 5 as one horse was withdrawn during these period and n =5

DEX: Descending doses and frequency of administration of IV dexamethasone as described in methods section

DEX/ENV: Descending doses of IV dexamethasone coupled with a change in environment.

ENV: Change in environment with IV injections of saline of equal volume and frequency as the appropriate dose of dexamethasone

BALFV: Amount of bronchoalveolar lavage volume retrieved in mls

TC: Total cell count based on cells per ml X volume.

%Neut: Percentage of neutrophils based on a cytospin differential count of 200 cells.

TNeut: Total neutrophils (x 10^6) as determined by %Neut x TC x .01

%Lymph: Percentage of lymphocytes based on a cytospin differential count of 200 cells

TLymph: Total lymphocytes (x 10^6) as determined by %Neut x TC x .01

%Mono: Percentage of monocytes based on a cytospin differential count of 200 cells

TMono: Monocytes per ml (x 10^6) as determined by %Mono x TC/ml x .01

a: Value differs significantly from pretreatment (sample 1) value within the same treatment group

b: Indicates that the values differed between two subsequent Sample times within the same Treatment group. The two sample points that differ are indicated by the letter "b" followed by a common number. For example, if in Treatment group 1, if there was a significantly different between Sample 3 and Sample 4, then each value will have "b1" next to the value for that Sample time.

c: Values collected at a Sample period differ significantly between two Treatment groups. Values that differ will be signified by the letter "c" followed by a number