5-3-2019

Sedated Versus Non-Sedated Methacholine Challenge for the Diagnosis of Airway Hyper-Responsiveness in Horses

Amy Catherine Lack

Sedated versus non-sedated methacholine challenge for the diagnosis of airway hyper-responsiveness in horses

By

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

Mississippi State, Mississippi

May 2019

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Title of Study: Sedated versus non-sedated methacholine challenge for the diagnosis of airway hyper-responsiveness in horses

Pages in Study: 38

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Pasture-associated severe equine asthma (EPA) is a progressive condition affecting horses in the southeastern United States. Pulmonary function testing with methacholine challenge (MC) provides a definitive diagnosis by eliciting airway hyper-responsiveness. Most horses require extensive conditioning to accept the instrumentation. Our hypothesis was that MC protocols designed to elicit airway hyper-responsiveness would yield equivalent results in the presence and absence of sedation. Sedated and unsedated MCs were performed on 8 EPA-affected horses, with each horse acting as its own control. Acepromazine was superior to xylazine/butorphanol, resulting in sedation and data collection. Based on American Thoracic Society guidelines, an acceptable ability to detect differences in lung resistance is less than a twofold difference in the provocative concentration of methacholine that elicited a 40% increase in lung resistance ($PC_{40}R_L$). Significant differences in $PC_{40}R_L$ were not detected. Validation of a sedation protocol for use in MC will expand the application of this diagnostic.

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to the entire SPARAO research lab for the early morning pulmonary function testing sessions and patience while this protocol was developed. Lastly, many thanks to the Mississippi State University whose financial support made this project possible.

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CHAPTER I

SEVERE EQUINE ASTHMA

Nomenclature

Severe equine asthma previously known as "heaves", recurrent airway obstruction, and chronic obstructive pulmonary disease, is a naturally occurring disease that predominantly affects middle-aged horses[1–6]. Two subtypes exist and are named based on the environment that exacerbates clinical signs: barn dust-associated severe equine asthma and pasture-associated (i.e., pasture asthma) severe equine asthma[2,4,7]. Barn dust asthma occurs in horses in temperate climates spending the majority of their time stabled[4]. Pasture asthma occurs in horses housed on pasture during the warm months in the southeastern United States and United Kingdom[7–9].

Clinical Signs and Disease Prevalence

Clinical signs of Severe Equine Asthma include increased respiratory rate and effort, nostril flare, coughing, and respiratory distress. Affected horses may adopt an extended neck posture, become inappetent, and suffer severe weight loss. The disease is chronic and progressive and early in the disease course can present with seasonal signs of nonspecific exercise intolerance that make definitive diagnosis difficult.

Equine pasture asthma (EPA) is a disease characterized by airway hyper-responsiveness, bronchoconstriction, smooth muscle hyperplasia, and goblet cell metaplasia with mucus accumulation and neutrophilic inflammation of the lower

respiratory tract[5,7,9–12]. Onset of clinical disease is associated with environmental changes including increases in temperature, dew point temperature, humidity, grass pollen, and mold spores[8]. Hypersensitivity to aeroallergens including molds and pollens is presumed based upon the temporal association of clinical disease with these allergens[1,7,8,13]. It is estimated that approximately 3-5% of horses in the southern United States are affected with EPA[8]. In the United Kingdom, it has been estimated that up to 50% of EPA affected horses also have clinical signs consistent with the barn dust asthma subtype but this observation is not congruent with the fact that barn dust-associated asthma is quite uncommon in the southeastern United States[12].

Common Diagnostics

The diagnosis of EPA is suspected when a history of warm season recurring respiratory compromise with intervening periods of cool season clinical remission is present along with evidence of neutrophilic inflammation in bronchoalveolar lavage fluid (BALF) or transtracheal wash (TTA)[8–10]. Transtracheal wash samples may contain up to 90% non-degenerate neutrophils and be mucopurulent in appearance[1,4]. However, concurrent pneumonia should always be ruled out. Endoscopic examination of these horses frequently reveals tracheal mucus[1]. More chronically affected horses may have a thickened carina and experience dynamic airway collapse during endoscopy[1]. A BALF neutrophil percentage of greater than 20-25% is consistent with a diagnosis of severe equine asthma[14]. However, a recent study suggests that not all horses with severe asthma meet or exceed the neutrophil threshold on airway fluid samples[15]. In this study the horses with the most clinically severe disease had BALF neutrophil percentages of <20%[15]. Bullone et al suggest that the severe small airway pathology and mucostasis

present in advanced disease contribute to an obstructive physiology. This results in an inability to sample these most affected airways and a falsely low neutrophil percentage[15]. Complete blood counts in clinically affected horses are frequently normal or may have mild non-specific changes including a mild mature neutrophilia and an increase in fibrinogen[9]. A recently described technique utilizing a histopathologic scoring system for endobronchial biopsy samples correlates well with impulse oscillometry but only during periods of clinical exacerbation[16]. These challenges coupled with the seasonal nature and complete remission of clinical signs highlight the limitations of many of the common diagnostics employed to diagnose severe equine asthma. Early diagnosis and appropriate intervention are necessary to delay disease progression.

CHAPTER II

PULMONARY FUNCTION TESTING

Conventional Pulmonary Mechanics

Pulmonary function testing in conjunction with methacholine challenge provides an objective method to document the presence, severity, and reversibility of airway hyper-responsiveness in horses[17,18]. This is particularly helpful for diagnosis during seasonal disease remission and early in the disease course when signs are mild. The classical method and current gold standard for pulmonary function testing is referred to as Conventional Pulmonary Mechanics and involves the insertion of an esophageal balloon attached to a catheter for measuring transpulmonary pressure[19]. Horses must wear a face mask that encompasses their muzzle to approximately the level of the infraorbital foramen for the duration of the testing. To ensure accurate measurements an elastic "girdle" attached to the mask must fit snugly against the horse's face and extends 3-4cm above the mask creating an air tight seal. A pneumotachograph is fitted to the mask to measure airflow. Measures of pulmonary function commonly acquired from conventional mechanics include resistance of the lung (R_L), dynamic compliance (C_{dyn}), elastance (E), and maximal change in pleural pressure (ΔPpl_{max}). Using these measurements, it is possible to monitor changes in pulmonary mechanics in real time both at rest and in response to a variety of drugs (methacholine, histamine, levalbuterol). Horses experiencing bronchoconstriction have an increase in R_L and ΔPpl_{max} and a decrease in

C_{dyn} and E. Though our research horses have been trained to pulmonary function testing using operant conditioning, not every horse has the temperament to accept the instrumentation while unsedated. As with many equine diagnostic procedures, the use of sedation could facilitate methacholine challenge in clinical patients. However, sedation has been demonstrated to alter airway caliber and decrease ventilation[20–23]. These effects call into question the validity of pulmonary function testing in the presence of sedation.

Impulse Oscillometry

An alternative method of pulmonary function testing is impulse oscillometry. This method was adapted for use in horses and is the preferred technique for non-compliant human patients[24]. A non-compliant patient is considered one that regardless of the reason cannot follow the directions to perform forced expiratory maneuvers[24]. Briefly, impulse oscillometry uses a high-frequency signal to determine respiratory impedance and is independent of respiratory rate[19]. As with conventional pulmonary mechanics, horses must wear an air-tight mask with a pneumotachograph throughout the testing protocol[19]. The primary variable from this system is impedance[25]. Impedance is determined by the resistance, elastance, and inertance of the respiratory tract in response to oscillatory frequencies[25]. In one study comparing the two techniques, impulse oscillometry was considered more sensitive[19]. However, the data obtained cannot be directly compared as the variables measured and resulting calculations are different[19].

Methacholine Challenge

Methacholine is a synthetic choline ester that acts as a non-selective muscarinic receptor agonist in the parasympathetic nervous system[26]. When inhaled, methacholine causes constriction of peripheral and central airways[26]. Methacholine along with carbachol, histamine, prostaglandin, and leukotrienes are considered direct stimulants for bronchoconstriction[26]. Direct stimulants initiate airway smooth muscle contraction by binding with local receptors (i.e. muscarinic, histaminic)[27–29]. Indirect stimulants include adenosine, bradykinin, metabisulfite, exercise, hypertonic aerosols, hypotonic aerosols, isocapnic hyperventilation, mannitol, and propranolol[26]. Indirect stimulants result in the activation of intermediate pathways[28,30]. Many of the end products of these pathways are inflammatory mediators which then elicit bronchoconstriction[28,30].

Bronchoprovocation with methacholine was first described in 1945[26]. The procedure is widely used in human medicine and has been employed in veterinary clinical medicine and as a research procedure to identify and quantify abnormal constriction responses of the airways[19,24,28,31]. In 2007 a revision of the 1999 ATS guidelines was published focusing on testing in non-compliant asthmatics, specifically toddlers[24,32]. In this article a greater than 40% increase in baseline lung resistance during a methacholine challenge was proposed as the end point for diagnosis of asthma[32]. Based on these recommendations Hunter *et al* demonstrated the utility of this end point in identifying horses with airway hyperresponsiveness[33]. This tendency, termed airway hyper-responsiveness, is a diagnostic feature of human asthma [24]. In human asthmatics, the degree of airway hyper-responsiveness correlates with disease severity[34–38].

Compliant asthmatics in human medicine are diagnosed via spirometry with methacholine challenge[24]. A baseline forced expiratory volume(FEV) is measured by spirometer and then methacholine is nebulized to the patient[24]. The FEV is acquired again after the dose of methacholine and another dose of methacholine administered[24]. This procedure is repeated until there is a > 20% decrease in FEV[24].

There are two protocols published and regulated by the American Thoracic Society for administration of a methacholine challenge: five-breath dosimeter protocol and two-minute tidal breathing protocol[24]. The five-breath technique requires inhalation of 5 doses of methacholine: 0.025, 0.25, 2.5, 10, and 25mg/ml[24]. Changes in pulmonary function are quantified following each dose. While in humans, the advantage of this protocol is the rapidity with which it can be completed, it necessitates the use of actuated nebulizers which have not been validated for horses[24]. Also, the large changes in dose concentration make it difficult to ascertain a specific provocative dose of methacholine[24]. The second method utilizes doubling doses of methacholine starting at 0.03-0.0625 mg/ml that are inhaled for two minutes during normal tidal breathing until a threshold of small airway constriction is achieved[24]. In human asthmatics, the dose of methacholine that elicits a 20% decrease in forced expiratory volume (PD_{20}) has been determined to be the most accurate method for quantifying the magnitude of airway hyper-responsiveness[24]. In noncompliant humans, who are unable to generate forced expiratory maneuvers, the dose of methacholine that elicits a 40% increase in lung resistance has been employed for evaluating airway hyper-responsiveness[32]. The achievement of a 40% increase in baseline resistance signifies the end point of this procedure. The final two concentrations of methacholine administered and the percent increase in resistance from

baseline to each of these doses is calculated. These values are then utilized to calculate the $PC_{40}R_L$ which represents the concentration of methacholine required to achieve a 40% increase in lung resistance. Employing the 40% threshold from noncompliant humans, in our laboratory, Hunter et al. demonstrated that horses with EPA reliably demonstrate airway hyper-responsiveness characterized by a 40% increase in baseline R_L at provocative concentration of methacholine $\leq$1 mg/ml[33]. Interestingly, this threshold has been previously considered diagnostic of moderate to serve human asthma (< 1 mg/ml)[24]. Like non-asthmatic humans, normal horses fail to respond to methacholine doses up to 8 mg/ml[17,24]

A challenge to the diagnosis of EPA is that affected horses undergo a period of disease remission during winter months when they appear clinically normal[9]. During remission, these horses are frustrating to identify. To address this dilemma, we have identified methacholine hyperresponsiveness in horses with EPA during disease remission (preliminary data Figure 1)[17]. This substantiates that methacholine challenge can be used as a diagnostic modality to identify horses with EPA regardless of clinical status. An impediment to performing methacholine challenge in horses, which is foundational to this work, is that few horses readily accept the necessary instrumentation for the 40-minute procedure.

CHAPTER III

EFFECTS OF SEDATION ON RESPIRATORY PHYSIOLOGY

Alpha-2 adrenoreceptor agonists

There are two types of adrenergic receptors, alpha and beta[39,40]. Alpha adrenergic receptors are further subdivided into α_1, α_{2A}, α_{2B}, and α_{2C}-[40]. α_2-adrenergic receptors are located pre and post-synaptically and mediate a variety of responses[40]. Agonism of pre-synaptic receptors results in vasodilation, decreased gastrointestinal motility, and inhibition of norepinephrine, epinephrine, and substance P[40]. Agonism of post-synaptic receptors causes sedation, analgesia, vasoconstriction, and inhibition of insulin release[40]. Centrally located α_2-adrenergic receptors in the locus coeruleus are responsible for the analgesia and sedation associated with administration of α_2-adrenergic agonist drugs[40,41]. The resulting effects on the cardiorespiratory system are bradycardia, hypotension, and bradypnea[40]. These parameters slowly return to baseline after administration[40].

The effects of the α_2-adrenergic agonist xylazine on pulmonary function testing with conventional mechanics were investigated in equine asthma affected and normal horses[20–22]. When administered to ponies in a barn-dust associated model exhibiting clinical signs of bronchoconstriction, xylazine decreased R_L and increased C_{dyn}. Changes in pulmonary function were not detected in control and in diseased ponies during clinical remission[20]. These findings indicate that xylazine dilates airways if bronchoconstriction is pre-existing.

Additional investigations have determined that increases in total airway resistance with xylazine are predominantly referable to increases in upper airway resistance and altered head position[21,22]. In a study comparing the airway dynamics of horses sedated with xylazine before and after placement of a nasotracheal tube, it was found that prior to placement of the tube, total airway resistance, upper airway resistance and pressure, respiratory work and pulmonary pressure were significantly increased compared to pre-sedation[21]. However, after placement of a nasotracheal tube in these same sedated horses, significant decreases in total airway resistance, upper airway resistance, and upper airway pressure were seen[21]. A study looking at the effects of head position after xylazine administration, on conventional pulmonary mechanics, reported an increase in pulmonary pressure, upper airway resistance, and lower airway resistance at low head position[22]. When the head position was neutral the resistance decreased significantly[22]. While this contrasts the effect of methacholine, which increases lung resistance predominantly in the lower airways, by inducing bronchoconstriction, lowering of the head position during sedation and associated decreases in lung resistance would confound the measurement of methacholine induced increases in lung resistance[42].

Only one previous study has reported the effects of detomidine alone on conventional pulmonary mechanics[43]. In this study, incremental dose escalation resulted in decreased dynamic compliance and respiratory rate[43]. Detomidine in combination with butorphanol was evaluated and resulted in decreased respiratory rate, minute volume, and maximal change in pleural pressure[23]. Dynamic compliance and lung resistance were not significantly different[23].

This indicates that α_2-adrenergic agonists have the potential to be relevant during methacholine challenge so long as head position, which has a large impact on upper airway resistance, is constant[22]. This would also suggest that methacholine is preferable to histamine for bronchoprovocation because histamine precipitates upper airway edema, which increases the contribution of upper airway resistance to total resistance[44].

Opioids

There are three major classes of opioid receptor: μ, κ, and δ[45,46]. Morphine is a pure μ-agonist[47]. Pure μ-agonists are uncommonly utilized in equine medicine due to central nervous system excitation and delayed gastrointestinal motility[47–49]. Butorphanol is commonly administered in horses and is a μ-antagonist and κ-agonist[47,49]. Drugs that provide sedation and analgesia via κ-agonism have less impact on gastrointestinal motility[48].

Butorphanol has not been evaluated as a sole agent for its effect on airway caliber in horses. When administered alone in standing horses butorphanol resulted in excitation without significant cardiopulmonary effects, including no alteration in respiratory rate[50]. Butorphanol is frequently combined with an α_2-adrenergic agonist to ameliorate CNS excitation[47]. Administration of α_2-adrenergic agonists potentiates the respiratory depressant effects associated with some opioids[47]. Morphine co-administered with xylazine caused a significant decrease in respiratory rate but not blood gas parameters in healthy, standing horses[51]. When butorphanol was administered in combination with the α_2-adrenergic agonist romifidine, Pa_{CO2} increased significantly, but no other blood gas parameters were affected[52]. Sedation with the combination of butorphanol, and the potent

α_2-agonist detomidine, decreased ventilation in both control and horses with recurrent airway obstruction, without altering measures of lower airway caliber (C_{dyn} together with R_L) nor tidal volume as measured by conventional pulmonary mechanics[23]. There was also a significant decrease in minute ventilation and ΔPpl_{max} in diseased horses relative to controls in this investigation[23]. However, in anesthetized dogs and rabbits instrumented for conventional pulmonary mechanics, butorphanol administration did not elicit significant differences from baseline resistance, predicting, congruent with the prior investigation, that butorphanol is unlikely to alter airway caliber in horses[53,54]. Cardiopulmonary effects of variable doses of buprenorphine, a partial agonist at the OP3 receptor, used in combination with xylazine were evaluated but its effects on airway caliber were not evaluated[55]. Respiratory rate was significantly decreased up to 45 minutes post drug administration but there were no significant changes in blood gas parameters[55].

Phenothiazine neuroleptics

Acepromazine is a phenothiazine neuroleptic tranquilizer that antagonizes central and peripheral post-synaptic dopaminergic receptors[21,2256]. Cholinergic, serotonergic, muscarinic, histaminic, and alpha-adrenergic receptors are variably antagonized[21,22,57]. The reticular activating system is depressed[21,22]. To date there are no studies describing the effects of acepromazine when administered alone on conventional pulmonary mechanics. In a 1988 study, respiratory curves were utilized as an indicator of respiratory depth based on the difference between central venous pressure during inspiration and expiration[58]. Acepromazine resulted in a significant decrease in respiratory rate and no

change in respiratory depth[58]. When xylazine was administered 20 minutes later the respiratory rate decreased again and the respiratory depth increased[58]. Acepromazine is frequently used in multi-drug protocols for sedation and anesthesia[58–61]. In these studies acepromazine minimally affected gas exchange parameters[58,61]. Acepromazine prior to upper airway endoscopy decreased abduction of the left arytenoid[62].

CHAPTER IV

EQUINE ESOPHAGEAL INNERVATION AND MOTILITY

The body of the esophagus consists of two muscular layers[63]. The outer muscular layer has longitudinal fibers and the inner layer has circumferential fibers[63]. The distal third of the equine esophagus is comprised of smooth muscle and the proximal two thirds is composed of skeletal muscle[63]. Three types of motility occur in the esophagus[64–66]. Primary peristalsis results from a stimulus originating in the oropharynx and upper esophageal sphincter[64,65]. Secondary peristalsis results from distention of the esophageal body, for example a remaining food bolus[64,65]. Tertiary peristalsis only occurs in smooth muscle[64,65]. Central and peripheral control mechanisms are responsible for esophageal motility with acetylcholine acting as the major stimulating neurotransmitter via alpha-adrenergic receptors[64,65,67]. The intrinsic plexi provide partial control of smooth muscle motility via innervation by the vagus nerve which receives input from the swallowing center[64,65]. Cholinergic influence is most prominent in the proximal smooth muscle and decreases distally[64,65]. The inherent latency gradient in the esophagus is decreased by cholinergic nerve stimulation[64,65]. Manipulation of this gradient changes the speed of peristalsis[64,65].

Contrast radiography studies revealed that detomidine but not acepromazine causes changes in esophageal motility[68,69]. In a study measuring esophageal manometric pressures xylazine/ butorphanol and detomidine significantly and dramatically decreased

the number of spontaneous swallows[70]. Acepromazine also decreased the number of swallows but less significantly[70]. The profound effect of xylazine/butorphanol and detomidine may have resulted from the accompanying CNS depression and reduction in response to normal mechanical and chemical swallowing triggers[70]. Detomidine and acepromazine increased the number of high-pressure events in the distal esophagus[70]. Xylazine and butorphanol increased the number of spontaneous and high-pressure events in the esophagus[70].

CHAPTER V

METHODOLOGY

Rationale

Several factors support the rationale that methacholine challenge will be valid in sedated horses. First, commonly employed sedatives (xylazine and detomidine) that exert mild bronchodilation during clinical exacerbation of recurrent airway obstruction are α_2 - adrenergic receptor agonists, whereas methacholine induces bronchoconstriction via muscarinic receptor signaling[1,7]. Second, methacholine challenge is routinely employed to demonstrate airway hyper-responsiveness in sedated and anesthetized animals[2-4]. Third, the methacholine challenge protocol employed determines the quantity of methacholine required to achieve a 40% increase in baseline airway resistance, effectively negating differences in baseline airway caliber and rate of methacholine inhalation caused by sedation[1].

Hypothesis and Objectives

The central hypothesis of this work is that methacholine challenge protocols that elicit a defined threshold change in airway caliber (relative to the baseline airway caliber) will yield equivalent results in the presence and absence of sedation[1]. This is irrespective of any increase or decrease in baseline airway caliber or decrease in the rates of methacholine inhalation caused by sedation. The first objective was to determine a

sedation protocol. The second objective was to compare the concentration of methacholine that elicited a 40% increase in R_L in sedated and unsedated horses with Equine Pasture Asthma. To address these objectives and increase the feasibility of methacholine challenge in clinical patients, we first performed a pilot study comparing the effect of combined xylazine and butorphanol to acepromazine alone, on the provocative concentration of methacholine that induces a 40% increase in lung resistance ($PC_{40}R_L$) using a two-minute tidal breathing protocol developed for methacholine challenge in noncompliant human asthmatics[1]. With the proper sedation protocol defined, we next addressed our primary aim: to compare the provocative concentration that causes a 40% increase in baseline pulmonary resistance in sedated and non-sedated horses. As changes in the provocative concentration for methacholine challenge less than two doubling doses are not considered clinically relevant in assessing the efficacy of human asthma therapeutics with methacholine challenge, we hypothesize that the $PC_{40}R_L$ in the sedated and unsedated state will be within two doubling doses of each other[11].

Animals

Eight adult horses previously diagnosed by methacholine challenge with Equine Pasture Asthma including four mares and four geldings were used in the study. Three of these horses were initially used in the pilot study to determine our sedation protocol. The procedure was repeated on all eight horses with the final sedation protocol. Each horse was determined to be in clinical remission by the primary investigator based on physical examination parameters and a clinical score of respiratory effort (CSRE) of ≤ 3[10]. Horses were brought in from pasture for the procedure and returned to pasture immediately after

data collection. No horse had been administered any medications for at least one month prior to use in this study. Procedures were performed with the approval of the Institutional Animal Care and Use Committee.

Pilot Study

Three horses were administered xylazine (0.1 mg/kg IV) and butorphanol (0.01 mg/kg IV) 15 minutes prior to instrumentation for conventional pulmonary mechanics. Data collection was successful in one of these horses and unsuccessful in two, due to increased esophageal peristaltic waves (Figure 1). These waves interfered with data collection and prevented completion of the methacholine challenge protocol. This sedation protocol was deemed unacceptable. A new protocol, utilizing acepromazine, was designed and tested in three horses. Acepromazine (.01mg/kg) was administered 15 minutes prior to instrumentation. All three horses successfully completed the methacholine challenge protocol, achieving $\geq$40% increase in lung resistance.

Conventional Pulmonary Mechanics Instrumentation

Horses were instrumented for the measurement of conventional pulmonary mechanics as previously described. The horse was measured from the nares to the 10-13th intercostal space and the distance marked on a rigid fluoropolymer catheter. An esophageal balloon was secured over this catheter which was passed through the nares to the level of the mark. The mark represented the thoracic esophagus between the heart and cardiac sphincter. The catheter-balloon unit was then placed in line with a pressure transducer

(DP 45-28, Validyne Corp) to monitor pleural pressure[7-10]. The balloon was uniformly filled with air across all tests to optimize pressure signals, which are initially recorded for 2-3 minutes to insure proper placement and pressure values consistent with the magnitude of respiratory effort. The catheter was manipulated to improve waveforms (absence of cardiac deflections and wave forms with negative deflection during inspiration), so the exact final location of the balloon was unknown. The balloon had to remain caudal to the heart as the ventricular motion interfered with pleural pressure waveforms. Flow was measured using a Fleisch #5 pneumotachograph and transducer (DP 45-14, Validyne Corp) fitted into a mask (Aeromask, Trudell International) that sealed to the horse's face and covered the nares, while minimizing dead space. Maximal change in pleural pressure (ΔPpl_{max}) and total pulmonary resistance ($R_{L)}$, and dynamic compliance (C_{dyn}) were measured as the average of 15 representative breaths using Buxco Systems Biosystems XA software (v. 2.5). Measurement of these parameters in horses has been well described,[7-10] and is technically straight forward in horses that are trained to accept the instrumentation.

Methacholine Challenge and Data Collection

Methacholine challenge was performed using the 2-minute tidal breathing protocol as described in guidelines by the American Thoracic Society with nebulized doses from 0.03125mg/ml – 8 mg/ml[1]. The nebulizer (Pari LC-Star reusable nebulizer) and air compressor (Pari ProNeb Turbo) were standardized for consistent administration. Baseline maximal change in pleural pressure (ΔP_{plmax}) and total pulmonary resistance (R_L), were determined from 15-20 breaths at rest and following nebulization of 0.9%

saline. A target of 40% increase in pulmonary resistance was calculated using the below equation (5.1) and doubling doses of methacholine were serially administered via nebulization in 5-minute intervals.

$$R_L \text{ Target} = (0.4 * R_L \text{ Baseline}) + R_L \text{ Baseline} \quad (5.1)$$

Between each methacholine dose, 15-20 representative breaths were recorded, and the challenge was discontinued at the methacholine concentration that increased R_L $\geq$40% above baseline R_L. During the methacholine challenge, the average R_L for each dose of methacholine was calculated from the data collected. This prevented horses from receiving additional methacholine doses after the target R_L was reached. Following methacholine challenge, 1.25mg of levalbuterol was administered via nebulization to reverse bronchospasm, which was confirmed by improvements in ΔP_{plmax}, R_L, and C_{dyn} at 7- and 15-minutes post nebulization.

Each horse underwent two methacholine challenges, one sedated and one unsedated, allowing each horse to act as its own control. For the sedated methacholine challenge, acepromazine (0.05-0.1mg/kg based on primary investigator's discretion) was administered intravenously 15 minutes prior to instrumentation. Following instrumentation, baseline data was collected and repeated in 2 minutes. The horse's head position and height were noted by two trained observers during baseline collection. The same observers monitored the horse's head position and height throughout the procedure. If the horse's head height and position changed during data collection, external stimuli were provided until the head returned to the baseline position (i.e. the head was not manually repositioned). The methacholine challenge was commenced as previously described with the final dose of methacholine causing $\geq$ 40% increase in baseline R_L.

Each horse was then nebulized levalbuterol to reverse methacholine induced bronchoconstriction.

Data Analysis

The last two concentrations of methacholine administered were used to manually calculate a $PC_{40}R_L$. $PC_{40}R_L$ = antilog [log C_1 + $\frac{(log\ C2 - log\ C1)(40 - R1)}{R2 - R1}$] (5.2)

This equation was modified from the equation utilized to calculate the FEV_{20}. In this equation C_1 represents the second-to-last methacholine concentration, C_2 the final concentration of methacholine (dose resulting in ≥ 40% increase in R_L from baseline), R_1 the percent rise in R_L after C_1, and R_2 the percent rise in R_L after C_2.

$(R_{LN} - R_{LB} / R_{LB}) * 100$ (5.3)

In the above modified percent increased equation, R_{LN} represents the measured resistance after administration of the ultimate or penultimate methacholine dose. R_{LB} represents the baseline resistance.

The $PC_{40}R_L$ of the unsedated and sedated methacholine challenges for an individual horse were compared. Under the American Thoracic Society guidelines, when comparing two $PC_{40}R_L$ from the same individual, if the results are within two double doses(4 times) of each other they are considered not significantly differently. The unsedated $PC_{40}R_L$ was used to establish the two doubling dose range in this study. A two-tailed T-test for 2 dependent means was performed with an $\alpha < .01$.

CHAPTER VI

RESULTS

Sedated and non-sedated pulmonary function testing and methacholine challenge were well tolerated by all horses. Each horse was monitored by the primary investigator for signs of sedation prior to instrumentation. Horses demonstrated a lower head position, relaxed ear posture, decreased response to environmental stimuli, slower gait, and hind limb toe dragging. No adverse effects to the sedative were observed.

Xylazine and Butorphanol Protocol

Three horses were sedated with xylazine and butorphanol. During baseline data collection, frequent, irregularly-shaped, tall, peaked, pressure waves occurred from the pleural pressure (i.e. esophageal pressure) catheter (Figure 6.1). These waves interfered with data collection. Two of the three methacholine challenges initiated under the xylazine and butorphanol protocol could not be completed due to these abnormal waves from the pleural pressure (i.e. esophageal pressure) catheter. Inability to collect the pleural (esophageal pressure) data precluded the use of xylazine and butorphanol sedation for methacholine bronchoprovocation.

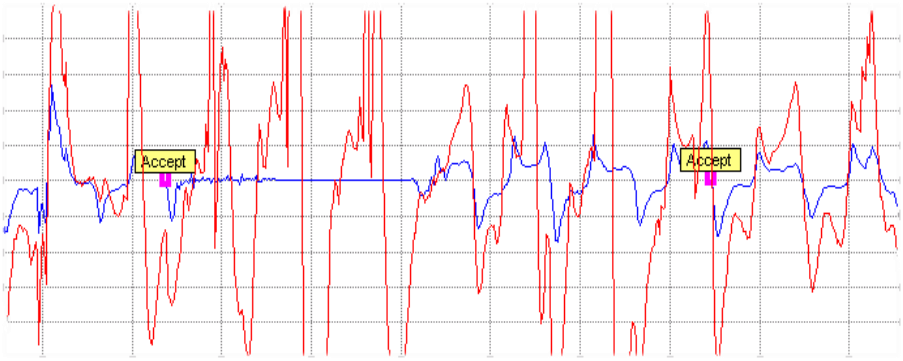

Figure 6.1 Sample baseline esophageal pressure and airflow waves from a horse with EPA that was sedated with xylazine and butorphanol.

The above image demonstrates intrapleural pressure (red) and airflow (blue) waves generated by Buxco X_a software from the baseline data of a horse sedated with xylazine and butorphanol. Each blue waveform is the equivalent of one respiratory cycle (inspiration and expiration). The red line should have a waveform appearance as indicated in the top panel of Figure 6.2 and the two waveforms should overlap for each breath. In this image there are pressure tracings that have no relationship to the respiratory cycle, including waves that are narrow and tall, and which have multiple oscillations which are not associated with changes in flow. These unusual waves precluded the ability to attain useful data. The waveforms were monitored for 45minutes with no change in frequency or character of the pressure tracing.

Acepromazine Protocol

All acquired data sets from 8 horses administered acepromazine sedation were complete and yielded pleural pressure waves that were in phase with flow. The abnormal pressure waves described under the xylazine and butorphanol protocol were infrequent and did not prevent data collection (Figure 6.2).

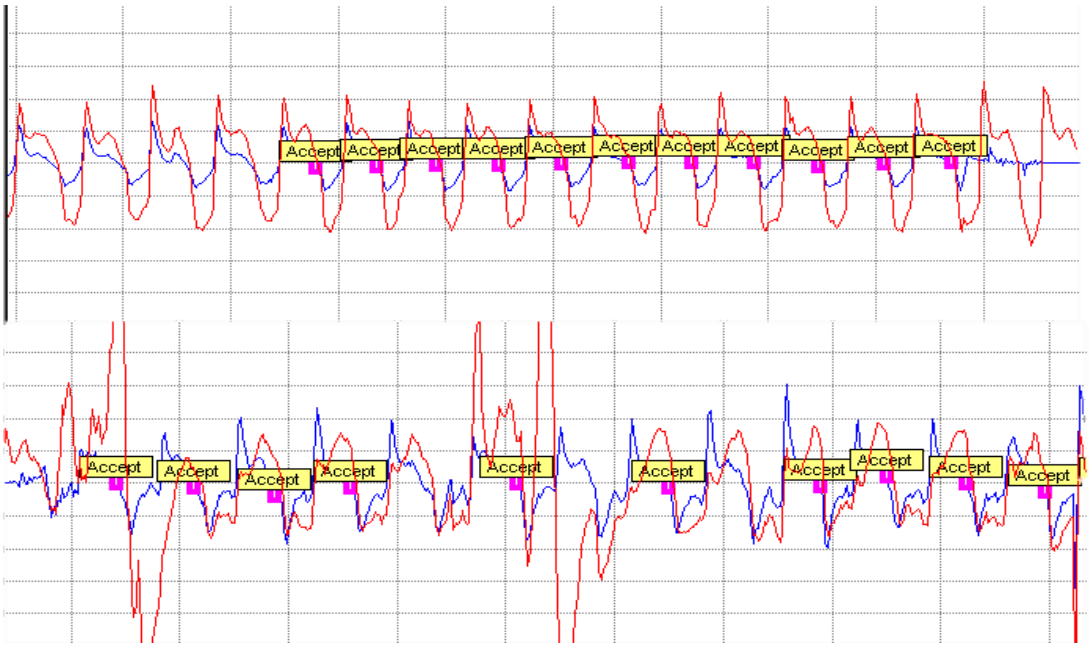

Figure 6.2 Pleural pressure and flow waves collected with the acepromazine protocol in a horse with EPA

The top image represents ideal wave forms during data collection. The flow (blue line) and intrapleural pressure (red line) tracings should overlap. The rise in esophageal pressure (positive deflection) and air flow correlates with expiration and the decrease (negative deflection) with inspiration. The small bump during the expiratory phase indicates an expiratory pause and is common in horses. The bottom image represents the infrequent abnormal pressure waves that occurred with acepromazine sedation but did not prevent data collection or the completion of a methacholine challenge

All eight horses showed a dose response to nebulization of doubling doses of methacholine. A representative response to methacholine challenge in a horse in the absence of sedation and again during sedation is presented in Figure 6.3. Consistent with the presence of airway hyper-responsiveness, all eight horses reached their target R_L at a concentration of methacholine less than 8mg/ml (Table 6.1). The highest dose of methacholine administered was 2mg/ml during a sedated challenge. Each horse was then confirmed to return to or below the baseline R_L after nebulization of levalbuterol.

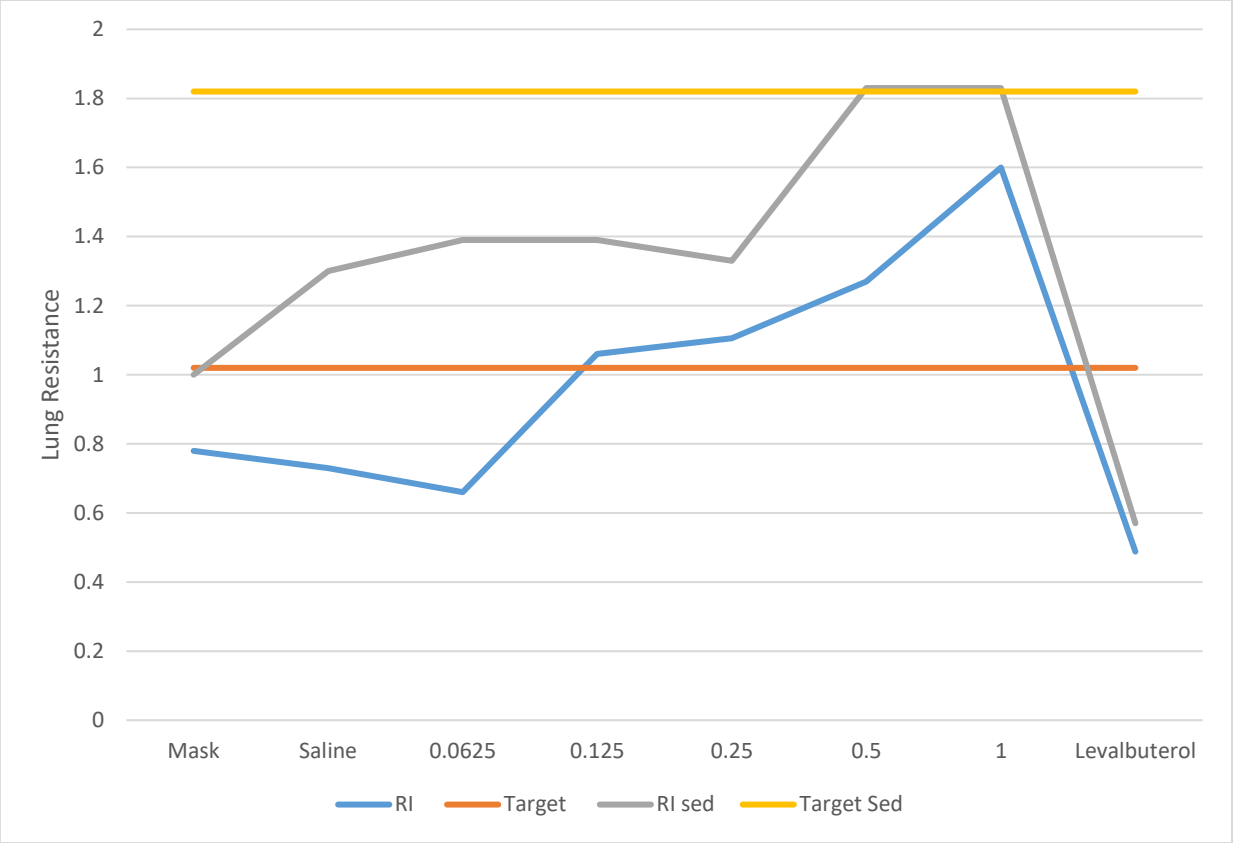

Figure 6.3 Sedated vs. non-sedated methacholine challenge comparison in a single horse with EPA

The x-axis represents the stages of the methacholine challenge. Starting with acquisition of a baseline, denoted as "mask", followed by nebulization of saline as a control, increasing mg/ml doses of methacholine until the target is reached, and lastly nebulization of levalbuterol. The y-axis provides the calculated average R_L associated with each challenge stage.

The sedated $PC_{40}R_L$ for each horse was within two doubling doses of the unsedated $PC_{40}R_L$ (Table 6.1 and Figure 6.4). Three of the horses had a sedated $PC_{40}R_L$ that was less than their unsedated $PC_{40}R_L$ and five horses had a $PC_{40}R_L$ that was greater than their unsedated $PC_{40}R_L$. The average and median unsedated $PC_{40}R_L$ were 0.19 and 0.175, respectively. The average and median sedated $PC_{40}R_L$ were 0.25 and 0.27,

respectively. The two tailed t-test with dependent means yielded a t value of 1.81. The value of p was 0.11. This supports the conclusion that the results of the two challenges were not significantly different.

Table 6.1 Sedated and unsedated $PC_{40}R_L$ from 8 horses with EPA

Horse	$PC_{40}R_L$ Acceptable Range	$PC_{40}R_L$ Unsedated	$PC_{40}R_L$ Sedated
1	0.090 – 1.440	0.360	0.350
2	0.030 – 0.480	0.120	0.280
3	0.038 – 0.600	0.150	0.100
4	0.013 – 0.208	0.052	0.068
5	0.050 – 0.800	0.200	0.430
6	0.060 – 0.960	0.240	0.330
7	0.035 – 0.552	0.138	0.201
8	0.068 – 1.080	0.270	0.260

Calculated $PC_{40}R_L$ for sedated and unsedated methacholine challenge are indicated for each horse tested. The acceptable range for $PC_{40}R_L$ is calculated by multiplying (high end) or dividing (low end) the unsedated $PC_{40}R_L$ by 4 or two doubling doses.

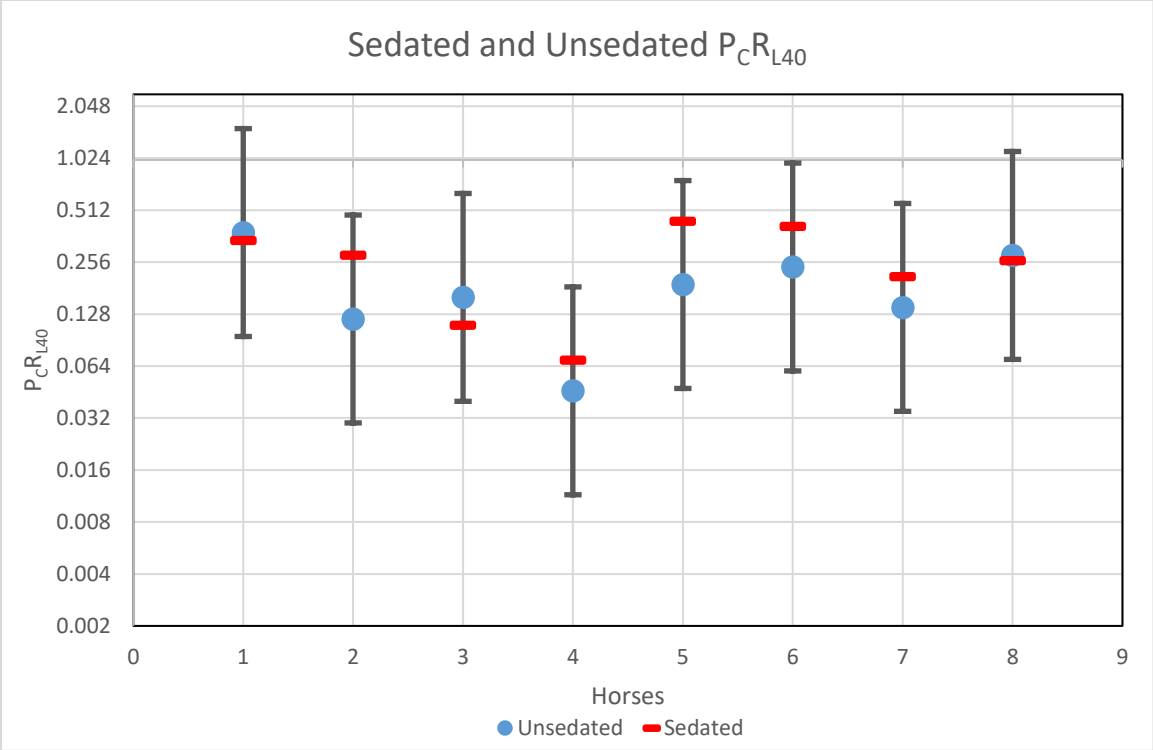

Figure 6.4 Comparison of sedated and unsedated $PC_{40}R_L$ from 8 horses with EPA

The x-axis represents the 8 individual horses used in the study. The y-axis represents $PC_{40}R_L$. The black bar indicates the allowed two-doubling dose range. The blue circles represent the unsedated $PC_{40}R_L$ and the red dash the sedated $PC_{40}R_L$.

CHAPTER VII

CONCLUSION

Discussion

Acepromazine provided adequate sedation, was well-tolerated by all horses, and did not interfere with data collection or results during pulmonary function testing with a methacholine challenge. The differences between the $PC_{40}R_L$ calculated from unsedated and sedated challenges were less than two doubling doses of each other which is not considered clinically relevant under the American Thoracic Society guidelines[24]. The American Thoracic Society is the organization responsible for providing gold standards for diagnosis of asthma in humans.

Horses presenting in seasonal remission or early in the disease, present a significant diagnostic challenge. Many of the commonly utilized diagnostics have non-specific or normal results under these circumstances. Identifying and initiating proper environmental and medical management as early as possible is key to delaying disease progression. Identification of horses at high risk of having pasture-associated severe equine asthma, based upon positive methacholine bronchoprovocation during the season of clinical disease remission, also provides an adjunctive testing modality for horses being considered for purchase in the southeastern United States during this time period. However, due to its progressive nature, need for radical management changes, and

frequent need for administration of systemic corticosteroids, this is not a diagnosis that can be made without consequence.

This validation of a sedation protocol for methacholine challenge in horses expands the population of horses who are candidates for this procedure. Previously, horses have needed extensive training and the right temperament to tolerate the instrumentation and testing environment. This limited the use of this diagnostic to extensively conditioned horses in small research herds. This study demonstrated the utility of sedated methacholine challenge, providing additional data proving that horses in seasonal remission maintain elicitable airway hyper-responsiveness. All the horses in this study met the target R_L after administration of $\leq$ 1mg/ml methacholine. Humans who respond at these low concentrations are categorized as having moderate-severe asthma[24].

Five of the eight horses required an additional dose of methacholine during the sedated challenge compared to the unsedated challenge. Respiratory rate depression is a common side effect of sedation in horses. We hypothesize that this caused these five horses to inhale a lower amount of methacholine during the prior steps of the tidal breathing protocol. However, congruent with our hypothesis, differences in the $PC_{40}R_L$ between sedated and non-sedated horses were within the two doubling doses, which is not considered to be clinically significant in evaluations of human asthma.

Another common side effect of sedation is a decreased head-to-ground height. This increases upper airway resistance and changes airway dynamics[22]. As anticipated, during this study, it was observed that as a horse's head lowered, R_L increased. If the horse was stimulated and returned to the original head position, R_L would return to the previous level. If the horse's head position became higher during the procedure, then R_L

decreased. The observed differences were significant and would dramatically change the target R_L for the methacholine challenge. Due to this observation, the horse's head position was marked during acquisition of baseline parameters. For the remainder of the procedure it was monitored continuously by two handlers to ensure it was consistent throughout the entire procedure.

Abnormal pressure waves, associated especially with the administration of xylazine and butorphanol, were unanticipated and severely affected the procedure, preventing useable data collection. The waves were persistent for 45 minutes after instrumentation, at which time the procedure was discontinued in two horses. After thorough review of the literature, it was hypothesized that this sedation protocol induced secondary esophageal peristaltic waves. The combination of xylazine and butorphanol has been shown to markedly decrease spontaneous swallowing[70]. This is most likely due to profound central nervous system depression and thus depression of the swallowing center[70]. When a food bolus remains in the body of the esophagus and cannot be cleared by primary peristalsis that is initiated by swallowing, secondary peristalsis is initiated[64,70]. It has been previously proposed that pooling saliva in sedated horses can be a trigger for secondary peristalsis [70]. In the current study, instrumentation for acquisition of intrapleural pressure required placement of an esophageal balloon in the thoracic esophagus. We hypothesize that this balloon may have provided the same sensory stimulus as a food bolus or pooling saliva resulting in peristalsis. Since the balloon was attached to a catheter that was secured to the horse's mask and immovable, the stimulus would have been continuous and have the potential to elicit the strings of high pressure waves that were observed. It is noteworthy that these waves were also observed after

administration of acepromazine but were infrequent. Additionally, acepromazine has been shown to only mildly decrease spontaneous swallowing in horses[70].

Limitations

The major limitation of this study is that it was performed in horses extensively conditioned to accept pulmonary function testing. Horses not accustomed to the instrumentation may require higher doses of acepromazine to safely perform the procedure. Since, secondary peristaltic waves were present in the horses in this study they may become more frequent with higher doses of acepromazine. This could limit the use of this diagnostic completely, if useable data cannot be collected. A higher dose could also impact the amount of methacholine needed to reach the target R_L and significantly alter the testing outcome. Future studies should be performed on horses with no prior experience with pulmonary function testing.

A second limitation of this study was that no normal horses were used in the population as a true control. While these horses could have provided useful information about the true effect of acepromazine on the parameters monitored during pulmonary function testing, the doses of methacholine necessary to achieve $PC_{40}R_L$ in normal horses are not known. This would be additionally useful as there is very little literature on the respiratory effects of acepromazine in horses as a sole agent. However, normal horses fail to respond to methacholine doses up to 8 mg/ml, a threshold congruent with non-asthmatic humans[33]. Accordingly, it is quite possible that doses of methacholine necessary to achieve $PC_{40}R_L$ in normal horses could be detrimental to human handlers.

The last significant limitation of this study was the small population size. The horses utilized in this study have advanced disease and may have introduced bias into the result. Given the wide range of disease severity, a larger population size that reflected the disease spectrum would provide additional merit to the current findings.

Conclusion

The present study is a useful addition to the Severe Equine Asthma literature as it provides a sedation protocol for use with conventional pulmonary mechanics and methacholine challenge. The $PC_{40}R_L$ comparisons provide the first evidence that it is possible to replicate the dose of methacholine that elicits a threshold level of airway contraction in the same unsedated and sedated horses. This not only expands the population of horses available to participate in methacholine bronchoprovocation in research studies, but also provides an alternative diagnostic modality for clinical cases with pasture-associated severe equine asthma that are being evaluated during in seasonal disease remission.

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