

2009

## Effects of clenbuterol on skeletal and cardiac muscle in horses

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**EFFECTS OF CLENBUTEROL ON  
SKELETAL AND CARDIAC MUSCLE  
IN HORSES**

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Interdepartmental Program in  
Veterinary Medical Sciences  
through the  
Department of Veterinary Clinical Sciences

by  
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August 2009

## ACKNOWLEDGEMENTS

The author would like to thank the members of her committee, Drs. Rebecca McConnico, Susan Eades and Dan Paulsen, for their patience and commitment to helping me complete this program successfully. Drs. McConnico and Eades were mentors in the clinic as well as contributing their time and ideas to this research project. Dr. Paulsen provided dedicated assistance in helping me find a suitable method to evaluate cell death in equine muscle.

This project utilized many different diagnostic modalities, and I am grateful for the technical assistance I received from each department. Within the EHSP, special thanks are due to Catherine Koch for introducing me to the world of research and for assistance with virtually all aspects of this project. Dr. Ann Chapman graciously and patiently contributed her expertise in echocardiography on multiple occasions. I thank Mike Keowen for helping me screen almost an entire herd of horses for this project, and for assisting with treadmill exercise, restraint and follow-up care for all the horses that actually qualified for the study. To Frank Garza, apologies are due for all the time you spent performing apoptosis assays! Thank you for your help with slide processing as well as horse handling and treadmill operation. I would also like to acknowledge the EHSP for funding the grant that supported this project.

For timely processing of serum clenbuterol samples, I am indebted to Dr. Steven Barker, Marian Waguespack and Connie David in the Louisiana State Racing Laboratory.

For sharing her lab space, time, reagents and immunohistochemistry expertise, special thanks are due to Julie Miller in the Immunohistochemistry lab. I also thank Hal Holloway & Cheryl Crowder in the Histology lab for processing muscle tissue sections, performing special stains on short notice, and teaching me how to coverslip. Additionally, I would like to thank Dr. Tim Morgan in the Department of Pathobiology for assistance in recovering tissue samples from the clinical cases of clenbuterol toxicity reported herein.

For performing (and helping me understand) statistical analyses, I would like to thank Dr. Giselle Hosgood and Michael Kearney.

For being my partner in life, crime, dancing and other things, I extend my love and gratitude to my husband, Miles Smith. Miles also provided an excellent editorial service for this manuscript, for which I'm sure my committee members were also grateful.

Last but not least, I wish to thank Dr. Jill Johnson for unfailing moral support, mentorship and some very good advice that finally spurred this project to completion.

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## LIST OF ABBREVIATIONS

AAEP	American Association of Equine Practitioners
ALP	alkaline phosphatase
AR	aortic root diameter
AST	aspartate aminotransferase
bpm	beats per minute
BUN	blood urea nitrogen
CLB	clenbuterol
CK	creatine kinase
cmH <sub>2</sub> O	centimeters of water
cTnl	cardiac troponin I
CVP	central venous pressure
dl	deciliter
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EF	ejection fraction
EIPH	exercise-induced pulmonary hemorrhage
FEI	Fédération Equestre Internationale
FS	fractional shortening
h	hour
IAD	inflammatory airway disease
IM	intramuscularly
IV	intravenously
IVSd	interventricular septal thickness, end-diastolic
IVSs	interventricular septal thickness, end-systolic
kg	kilogram
L	liter
lb	pound
LSU VTH&C	Louisiana State University Veterinary Teaching Hospital & Clinics
LVIDd	left ventricular internal diameter, end-diastolic
LVIDs	left ventricular internal diameter, end-systolic
LVPWd	left ventricular posterior wall thickness, end-diastolic
LVPWs	left ventricular posterior wall thickness, end-systolic
µg	microgram
µl	microliter
mEq	milliequivalent
mg	milligram
ml	milliliter
ng	nanogram
PA	pulmonary arterial diameter
pg	picogram
PO	by mouth
PT	prothrombin time
PTT	partial thromboplastin time
PRN	as needed
PSSM	polysaccharide storage myopathy
q	every
RAO	recurrent airway obstruction
RER	recurrent exertional rhabdomyolysis
sec	seconds
SQ	subcutaneously
U	units
USDA	United States Department of Agriculture
USEF	United States Equestrian Federation

## ABSTRACT

Clenbuterol is a commonly prescribed  $\beta_2$ -adrenergic agonist approved for veterinary use as a bronchodilator in horses with reactive and obstructive airway disease. Potential for abuse of this drug in the horse industry is substantial, due to the perceptions that clenbuterol increases performance and lean muscle mass. Although anabolic effects have been confirmed in multiple species, recent studies into the effects of clenbuterol in exercising horses suggest that clenbuterol doses within therapeutic ranges negatively impact aerobic capacity and cardiac function. Results of studies in murine models demonstrate that clenbuterol directly induces skeletal and cardiac muscle cell death at high doses.

Three cases of equine clenbuterol overdose are described, in which clinical signs of toxicity included tachycardia, muscle tremors, sweating and colic. Major laboratory abnormalities included hyperglycemia, azotemia and elevated creatine kinase activity. Two horses were euthanized due to complications of toxicity. Post-mortem abnormalities included skeletal and cardiac muscle necrosis.

The experimental study reported here examined the effects of oral clenbuterol on skeletal and cardiac muscle in clinically healthy horses undergoing treadmill exercise, as compared with a control group. Additionally, serum clenbuterol concentrations were measured throughout the treatment period.

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee. Twelve clinically healthy Thoroughbred horses between the ages of 3 and 10 years old were randomly assigned to either the control group (n=6) or the clenbuterol group (n=6). Animals in the control group received saline by mouth twice daily for 14 days. Horses in the clenbuterol group received clenbuterol by mouth twice daily for 14 days, at incrementally increasing doses up to 3.2 $\mu$ g/kg. Horses were subjected to daily submaximal treadmill exercise during the treatment period. Muscle biopsies were collected before and after treatment for determination of apoptosis. Echocardiographic measurements, serum clenbuterol concentration, and serum activities of creatine kinase, aspartate aminotransferase and cardiac troponin I were measured before, during, and after treatment. Venous blood samples were collected from the jugular vein(s) every 3 days during treatment. Echocardiography was repeated every 7 days after beginning treatment.

Serum biochemical and echocardiographic response variables were summarized as median and range. These variables were compared between treatment groups and across time periods using a

Mann-Whitney U test and Friedman's test for repeated nonparametric data, respectively. An adjusted level of significance at  $p < 0.01$  was used to reduce type I error. The presence of apoptosis in muscle biopsy samples was compared using a Cochran-Mantel-Haenszel stratified analysis, with a significance level of  $p < 0.05$ . Serum clenbuterol concentrations and percent apoptosis were summarized as mean  $\pm$  standard deviation.

No significant effect of clenbuterol or exercise on response variables was found between treatment and control groups at any time point, nor within groups over time. Clenbuterol was detected in the serum of all clenbuterol group horses during the treatment period. This study did not demonstrate any adverse effects of a 2-week course of clenbuterol treatment on equine cardiac or skeletal muscle at approved doses for treatment in horses.

## CHAPTER 1. INTRODUCTION

### 1.1 General Introduction

The use of performance-enhancing substances among human and equine athletes has long been the subject of intense scrutiny and regulation. Clenbuterol is one such substance that enjoys a unique place in sports medicine; despite its potential for abuse, it has been shown to be beneficial in the treatment of equine recurrent airway obstruction (RAO) and inflammatory airway disease (IAD)<sup>1</sup>, and it may slow the progression of disuse atrophy in humans<sup>2,3</sup>.

Clenbuterol is approved for use in non-food producing horses for treatment of respiratory disease involving the small airways, when administered orally at 0.8µg/kg to 3.2µg/kg twice daily for up to 30 days. Beneficial effects pertinent to the treatment of small airway inflammation include bronchodilation of small airways and enhancement of mucociliary clearance. Although clenbuterol is classified as a  $\beta_2$ -adrenergic agonist, its selectivity is only moderate, meaning that  $\beta_1$  and  $\beta_3$  receptors are also activated, albeit at a lower frequency. Additionally, the distribution of  $\beta_2$  receptors in multiple organ systems results in a wide range of effects on the body as a whole.

Initial safety studies required for USDA approval indicated that side effects in horses were transient and relatively mild (NADA 140-793); however, numerous recent investigations have documented negative effects of clenbuterol on athletic performance<sup>4-7</sup>. Results of studies in laboratory animals suggest that the overall response of skeletal and cardiac muscle to clenbuterol may be partially mediated through a dose-dependent induction of myocyte cell death<sup>8-12</sup>. Skeletal muscle appears to be more susceptible to the myotoxic effects of clenbuterol<sup>9,12</sup>.

Because clenbuterol is routinely administered to performance horses for reasons other than respiratory illness, and these horses are typically not under veterinary supervision for adverse side effects, closer investigation into the potential risks of clenbuterol use among equine athletes not suffering from restrictive airway conditions is warranted. No studies to date have specifically examined the effects of therapeutic doses of clenbuterol on skeletal myocyte cell death in the horse or its relation to serum clenbuterol concentration and common biochemical markers of muscle injury, such as creatine kinase (CK) and aspartate aminotransferase (AST). Additionally, the duration of treatment required to produce alterations in the echocardiogram is unknown.

## 1.2 Hypotheses and Study Objectives

The purpose of the study reported here is to report 3 cases of clenbuterol toxicity in the horse, and to determine if skeletal or cardiac muscle injury and/or cell death occurs in healthy, exercising horses receiving clenbuterol at doses normally prescribed for treatment of respiratory disease. The mechanisms by which clenbuterol may negatively affect aerobic performance appear to be multi-factorial and are not well-elucidated. Potential mechanisms pertinent to this work are induction of myocyte necrosis and remodeling of muscle tissue via initiation of apoptosis.

The first objective of this study is to report 3 cases of clenbuterol toxicity in the horse with documented tissue and serum clenbuterol concentrations, and to report the occurrence of skeletal and cardiac muscle injury in 2 of these horses.

The second objective is to determine if clenbuterol administration at normally prescribed doses is associated with an increase in apoptosis or necrosis in equine skeletal muscle. The working hypothesis is that the frequency of apoptosis will be significantly higher in horses administered clenbuterol for 14 days than in saline-treated controls.

The third objective is to determine if changes in cardiac function can be induced during a 14-day treatment with clenbuterol. Working hypotheses are that changes in left ventricular measurements will be present in all horses after exercise conditioning, and that measurements will be significantly different in horses receiving clenbuterol, compared with controls.

The final objective is to determine if common biochemical markers of skeletal and cardiac muscle injury are useful diagnostic tools for the detection of clenbuterol-induced skeletal or cardiac muscle injury. The working hypotheses are that activities of CK, AST and cardiac troponin I (cTnI) will be significantly higher in clenbuterol-treated versus control group horses, and that elevations in enzyme activities will be associated with histologic, echocardiographic or clinical evidence of muscle injury.

## CHAPTER 2. LITERATURE REVIEW

### 2.1 Role of Clenbuterol in Veterinary Medicine

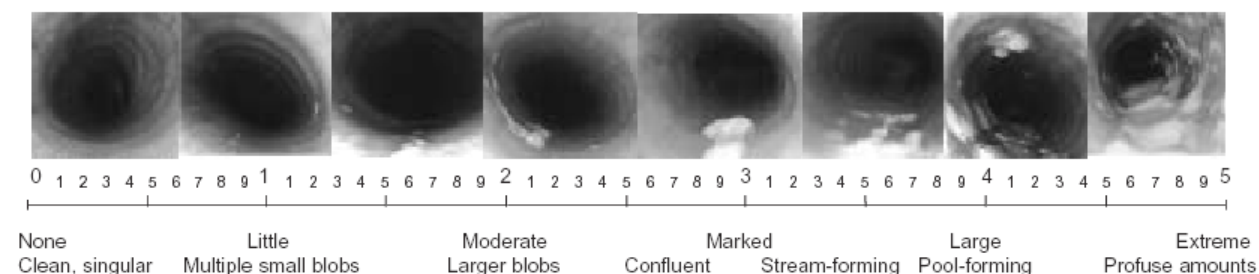
#### 2.1.1 *Therapeutic Use in the Horse*

Clenbuterol has been licensed in the United States since 1998 for use in the treatment of lower respiratory disease in horses (NADA 140-793), where bronchodilation and enhancement of mucociliary clearance are desired effects. The two most common disease syndromes for which clenbuterol is prescribed are recurrent airway obstruction (RAO) and inflammatory airway disease (IAD), although it may be used as an adjunct treatment in cases of bronchopneumonia. RAO and IAD are both considered syndromes of airway hyperreactivity, resulting in impairment of blood-gas exchange by excessive tracheal and small airway mucus accumulation, neutrophilic airway inflammation and bronchoconstriction.

RAO typically affects an older population of horses and is seasonally progressive without careful environmental management. Neutrophilic inflammation, increased airway secretions, bronchoconstriction and thickening of the bronchiolar walls produce the classic clinical signs of increased respiratory rate, increased respiratory effort, coughing and exercise intolerance. Despite periods of remission, airway remodeling further contributes to respiratory compromise over time, including respiratory epithelial metaplasia, peribronchiolar infiltrate and bronchiectasis. Acute episodes of RAO may be reliably triggered by exposure to hay molds or pasture antigens, and significant elevations in normal intrapleural pressure changes accompany clinical signs of RAO, as opposed to horses afflicted with IAD<sup>13</sup>. Forty-two to seventy-five percent of horses affected with RAO that responded to atropine challenge also responded to clenbuterol, based on either remission of clinical signs or reduction in intrapleural pressure change (NADA 140-973). Because of the progressive nature of RAO, long-term or repeated therapy with bronchodilators is often required to manage symptoms, especially during respiratory crises.

Conversely, IAD is a disease of younger horses in which pleural pressure changes and airway remodeling are not observed. The disease is commonly diagnosed in performance horses and may result from post-infective airway hypersensitivity associated with outbreaks of viral and bacterial respiratory disease in large training facilities<sup>14</sup>. Estimates of prevalence in performance horses range from 14% in young British racehorses<sup>15</sup>, up to 70% in poorly-performing National Hunt horses<sup>16</sup> and 74% among Japanese racehorses evaluated for poor performance<sup>17</sup>. The disease often reduces performance

and is characterized by alterations in tracheal mucus quantity, which have been scored from 0 to 5 using the tracheal mucus scoring system in Figure 1<sup>18</sup>. Mucus scores of 2 or greater are associated with poor racing performance<sup>19</sup>. Tracheal secretions contain increased numbers of neutrophils, and mast cells are frequently present<sup>13</sup>, although total inflammatory cell counts are lower than those of horses affected with RAO.



**Figure 1.** Scoring system for tracheal mucus, based on endoscopic observation.

### **2.1.2 Potential for Abuse**

Clenbuterol abuse has been reported in humans and most livestock species, where it is used to increase lean muscle mass. Use in livestock was prohibited after multiple cases of human toxicosis resulted from ingestion of clenbuterol residues in muscle and liver<sup>20-23</sup>. In horses, clenbuterol is used outside a valid veterinary-client-patient relationship due to an industry perception that it is a non-specific performance-enhancing drug. In addition, clenbuterol has been advocated for the treatment of other performance-limiting respiratory conditions, most notably exercise-induced pulmonary hemorrhage (EIPH). Objective evaluation of its value in prevention of EIPH failed to demonstrate any benefit<sup>24</sup>.

### **2.1.3 Regulation of Clenbuterol in the Horse Industry**

Clenbuterol is tightly regulated in the US flat racing industry, and specific withdrawal times for the drug prior to racing are established by each racing commission. Detection of the drug above allowable concentrations in the urine or blood post-race can result in disciplinary action against the horse's trainer or owner. English performance horses participating in FEI and USEF-sanctioned events are strictly prohibited from receiving clenbuterol; the animal must be withdrawn from competition even if the drug was given under a veterinarian's direction. However, client access to large quantities of clenbuterol is perpetuated because of the relatively high frequency of IAD in the performance horse population, for which clenbuterol is a popular treatment. Additionally, use of clenbuterol is not regulated in many



disciplines, nor is it uniformly regulated at all levels of competition. These situations further engender questionable use of clenbuterol within the horse industry, i.e. administration outside of a valid veterinary-client-patient relationship.

## **2.2 Pharmacology**

### **2.2.1 Pharmacokinetics and Tissue Distribution**

Clenbuterol is rapidly absorbed in less than 15 minutes following oral administration with a bioavailability of 83%<sup>25</sup>. With twice daily administration of 1.6µg/kg clenbuterol by mouth, half-life was calculated to be 12.9 hours, and mean steady-state serum concentrations of 508.1pg/ml were achieved after 48 hours<sup>25</sup>. Clenbuterol is metabolized in the liver and is primarily excreted in the urine, with a small percentage (<5%) excreted in the feces. Urine concentrations are 100 times greater than plasma concentrations, and clenbuterol may be detectable in the urine for up to 12 days<sup>25</sup>. Prolonged urinary excretion may be due in part to clenbuterol distribution in the tissues; tissue clearance (elimination half-life is >21 hours for all tissues) is prolonged relative to serum clearance<sup>26</sup>. The liver, lung and left ventricle contain the highest concentrations of clenbuterol. Following 2 weeks of clenbuterol administration, tissue/plasma ratios of clenbuterol concentration in the liver (42.2), eye fluids (21.5), lung (9.9) and left ventricle (7.2) were significantly elevated 72 hours after administration of the final dose<sup>26</sup>. Clearance from the pectoralis muscle was fastest of all tissues sampled in this study.

### **2.2.2 Distribution of $\beta$ -Adrenoreceptors in the Horse**

The multi-systemic effects of clenbuterol are mediated by the distribution of  $\beta$ -adrenoreceptors in the body. It is considered a moderately selective  $\beta_2$ -adrenergic agonist with dose-dependent effects on multiple organ systems.  $\beta_2$  adrenergic receptors are present in skeletal and cardiac myocytes, hepatocytes, sweat glands, nervous tissue, respiratory smooth muscle, vascular smooth muscle, uterus, and ileum<sup>1,12,27-30</sup>.  $\beta_1$  adrenoreceptors are present in the heart and may be activated directly with high doses of clenbuterol, or secondarily via release of norepinephrine from  $\beta_2$ -stimulated sympathetic neurons. Clenbuterol is also reported to activate  $\beta_3$  (or “ $\beta$ -atypical”) receptors, which are purportedly responsible for the lipolytic and other repartitioning metabolic effects of clenbuterol, and which are relatively insensitive to blockade by the majority of  $\beta$ -adrenergic antagonists, including propranolol<sup>1,27</sup>.

### **2.2.3 Effects at the Cellular and Molecular Level**

$\beta_2$  adrenoreceptors are coupled to G proteins that mediate a vast array of second-messenger signaling pathways<sup>31</sup>. In the uterus, ileum, vascular smooth muscle and respiratory smooth muscle,  $\beta_2$  receptors generally mediate smooth muscle relaxation through adenylyl cyclase-mediated inhibition of acetylcholine release. In the liver, gluconeogenesis is induced.

Norepinephrine is released from post-synaptic sympathetic cardiac neurons in response to  $\beta_2$  activation<sup>28</sup>. Positive inotropic and chronotropic effects on the cardiac muscle are mostly mediated by the action of norepinephrine on cardiac  $\beta_1$  receptors. However,  $\beta_2$  adrenoreceptors comprise approximately 30% of the receptor population of the atrial myocardium and 18% of the ventricular myocardial receptors in the horse<sup>32</sup>. At sufficiently high doses, cardiac  $\beta_1$  receptors may also be activated directly by clenbuterol.

In skeletal muscle,  $\beta_2$  stimulation results in alterations of intracellular calcium content<sup>33</sup>, which may result in either increased contractile force or cell death. Clenbuterol has been studied extensively in human medicine as a potential therapeutic agent for diseases of muscle wasting, such as disuse atrophy, cachexia or decreased cardiac load in left ventricular assist device support<sup>3,34-36</sup>. Recent research has indicated that clenbuterol may inhibit protein degradation in skeletal muscle by G protein-mediated inhibition of calpain activation and ATP-dependent ubiquitination<sup>35</sup>. Clenbuterol may also modulate the ubiquitin-proteasome pathway by a mechanism unrelated to  $\beta$ -adrenoreceptor activation<sup>2</sup>. Specific effects of  $\beta_2$  stimulation on skeletal and cardiac muscle are discussed further in the sections to follow.

## **2.3 Adverse Effects and Toxicity**

### **2.3.1 Clinical Signs of Toxicity**

Toxic effects of clenbuterol may be generally considered to be caused by excessive sympathetic stimulation. During safety trials in horses, the most common side effects were sweating (27.6%), muscle tremors (18%) and anxiety (2.8%). Signs were more profound at higher doses, but all resolved without intervention in a few hours. Reported cases of clenbuterol toxicity in humans describe sinus tachycardia, myalgia, gastrointestinal upset, and hypokalemia as the most common clinical signs<sup>20-23,37-39</sup>. Supraventricular arrhythmias have also been reported in association with clenbuterol ingestion in people<sup>40</sup>. The recommended treatment of clenbuterol toxicity in humans is administration of a  $\beta$ -

adrenergic antagonist (i.e., propranolol or esmolol) and correction of glucose and electrolyte abnormalities<sup>37,38,40,41</sup>. Repeated administration of  $\beta$ -adrenergic antagonists is frequently required<sup>37,38</sup>, as toxic effects can last up to 6 days<sup>39</sup>.

### **2.3.2 Species Variation in Toxicity**

Mice and other livestock species appear to tolerate significantly higher doses of clenbuterol than horses with no demonstrable side effects. Doses reported to induce “repartitioning” in cattle are as high as 30 $\mu$ g/kg<sup>1</sup>. In numerous murine experiments, clenbuterol was administered at doses from 10 $\mu$ g/kg up to 2mg/kg/day in both brief, terminal experiments and long-term exercise physiology studies<sup>4,8-10,33,42,43</sup>. Interestingly, although muscle injury was documented in rats receiving clenbuterol<sup>12</sup>, no clinical signs of toxicity were described in any murine study. In contrast, equine and human reports of toxicity are numerous. In Chapter 3, sustained tachycardia, muscle tremors, and skeletal and cardiomyocyte necrosis were described in a horse that received an estimated dose of 10 $\mu$ g/kg clenbuterol. Similarly, reported cases of human toxicity describe the occurrence of toxicosis with ingestion of clenbuterol doses between 0.9 $\mu$ g/kg<sup>20</sup> and 4.8mg/kg<sup>37</sup>. This apparent discrepancy may be explained as failure to recognize these clinical signs in rodents; alternatively, it may suggest that there is a wide species variation in sensitivity to clenbuterol, with horses being exquisitely sensitive to  $\beta$ -adrenergic stimulation.

### **2.4 General Effects on Performance**

Although the primary reason for clenbuterol abuse is, generically speaking, performance enhancement, results of investigation into objective measures of performance enhancement are mixed. Multiple studies agree that clenbuterol enhances lean muscle mass<sup>3,10,33,42,44-46</sup>, but whether this translates into increased muscular strength is controversial. Conversely, a majority of investigations also suggest that clenbuterol has a negative effect on endurance and exercise tolerance<sup>4-7</sup>. Decreased performance on a standardized exercise test was observed in Standardbred mares receiving 2.4 $\mu$ g/kg clenbuterol for 8 weeks<sup>5</sup>. When treadmill velocity was measured as a function of heart rate, Arabian horses administered a single 0.8 $\mu$ g/kg intravenous dose of clenbuterol 30 minutes prior to treadmill exercise were unable to achieve comparable speeds to control horses<sup>7</sup>. An increased incidence of sudden death from cardiac failure was also observed in one study of exercising rats receiving clenbuterol<sup>4</sup>. The specific mechanism(s) by which clenbuterol exerts these effects are still unclear.

## **2.5 Effects on the Musculoskeletal System**

### **2.5.1 Increase in Lean Muscle Mass**

Clenbuterol causes an increase in lean muscle mass in all mammalian species studied. This effect is one of the primary reasons for its abuse in human body-builders and food-producing animals. It is partly mediated through a relative loss of fat mass in conjunction with muscular hypertrophy, a phenomenon known as repartitioning. Clenbuterol may promote lipolysis through its action on  $\beta_3$  receptors in adipose tissue. Additionally, increased serum concentrations of adiponectin and decreased concentrations of leptin have been reported in horses receiving clenbuterol<sup>47</sup>. In another study of Standardbred mares receiving 2.4 $\mu$ g/kg clenbuterol for 8 weeks, total body weight increased by approximately 4%, while ultrasonography was used to demonstrate a decrease in rump fat thickness<sup>44</sup>. Interestingly, in this same study, exercise appeared to attenuate the gain in muscle mass (fat free mass) in horses administered clenbuterol, as opposed to horses receiving clenbuterol alone. In rats, administration of clenbuterol reliably increases skeletal and cardiac muscle mass by 11-29%<sup>4,10</sup>. Although an increase in the collagen weight (fibrosis) of skeletal and cardiac muscle may also contribute to the overall increase in muscle weight<sup>4,10</sup>, true cellular hypertrophy also occurs<sup>10,42</sup>.

### **2.5.2 Effects on Skeletal Muscle Strength**

Tensile strength in skeletal muscle can be measured and expressed in various ways; the methodology of choice often determines whether an effect of clenbuterol will be reported. Generally, if muscle groups are considered as a whole, an increase in strength is noted. In one study of human volunteers, there was a trend towards higher maximal voluntary strength in subjects receiving clenbuterol versus placebo<sup>3</sup>. This effect is likely due to increased muscle mass or hypertrophy, as it is seldom reported when peak force is adjusted for muscle weight or cross-sectional area. In rats receiving 2mg/kg/day clenbuterol for 2 weeks, an increase in overall leg muscle force was demonstrated using electrostimulation; however, no effect was seen when data were adjusted for the increase in muscle mass<sup>42</sup>. Conversely, long-term clenbuterol administration in horses did result in generation of increased force per cross-sectional area in the gluteus medius muscle<sup>48</sup>.

### **2.5.3 Alteration of Skeletal Muscle Fiber Composition**

Some of the physiologic effects of clenbuterol may be due to a shift in myosin heavy chain composition of skeletal muscle. Myosin heavy chains are categorized as type I, type IIA and type IIX. Clenbuterol induces a shift towards type II (specifically type IIX) myosin heavy chain fibers, which normally predominate in “fast-twitch” muscles<sup>42,45</sup>. This effect is most pronounced in “slow-twitch” muscles such as the soleus, in which type I heavy chains constitute the majority of fibers, and where the greatest concentration of  $\beta$ -adrenoreceptors is present<sup>49</sup>. In rats, these muscles undergo the most changes in dry weight, tensile strength and percentage of cell death in response to clenbuterol administration<sup>2,8,10,42,43</sup>. This effect may be due in part to the failure of slow-twitch muscles to undergo  $\beta_2$  receptor tachyphylaxis, as occurs in fast-twitch and cardiac muscle<sup>50</sup>.

Since aerobic conditioning through exercise produces a shift in muscle fiber composition from type IIX to type IIA or type I fibers, Beekley *et al* proposed that the clenbuterol-induced fiber composition shift towards type IIX directly reduces aerobic capacity<sup>45</sup>. Others have attributed decreased aerobic capacity to suppression of intracellular phosphofructokinase activity associated with clenbuterol administration<sup>42</sup>.

### **2.5.4 Modulation of Skeletal Muscle Cell Death**

A differential effect of clenbuterol dose on the type of skeletal muscle change has been quantified in the rat. Briefly, low doses induce muscular hypertrophy, and moderate-to-high doses ( $\geq 10\mu\text{g}/\text{kg}$ ) result in skeletal muscle apoptosis and necrosis<sup>8</sup>. The highest frequency of apoptosis (5.8%) was observed in the soleus muscle 4 hours post-clenbuterol administration<sup>8</sup>, and necrosis (4.4%<sup>12</sup> to 7.4%<sup>8</sup>) was most severe in the soleus 12-15 hours after a single administration. No effect was observed in the anterior tibialis muscle<sup>12</sup>, which is a fast-twitch muscle.

When clenbuterol was administered to rats once a day for 14 days, higher doses ( $\geq 100\mu\text{g}/\text{kg}/\text{day}$ ) were required to induce detectable myocyte apoptosis, and the frequency of apoptotic cells (0.15% to 0.3%) was lower than previously reported<sup>10</sup>. The apparent decrease in myotoxicity over time may be due to  $\beta$  receptor downregulation. Administration of  $10\mu\text{g}/\text{kg}$  clenbuterol every other day failed to induce myocyte apoptosis after 8 days; however significant necrosis occurred for up to 16 days<sup>8</sup>. Cell death is likely induced via signaling pathways downstream of the  $\beta_2$  receptor, as myotoxicity can be

prevented in skeletal muscle by pre-treatment with  $\beta_2$ -specific adrenergic antagonists, but not with  $\beta_1$ -adrenergic antagonists or norepinephrine depletion<sup>12,28</sup>.

Conversely, clenbuterol has been investigated in human medicine for its ability to slow the progression of disuse atrophy<sup>2,35,43</sup>. This effect has not been uniformly reproducible in all models. The implications of these findings for horses with pre-existing myopathies remain uncertain.

## **2.6 Effects on the Cardiovascular System**

### **2.6.1 Changes in Hemodynamics**

Clenbuterol lowers blood pressure by causing  $\beta_2$ -mediated relaxation of vascular smooth muscle, thereby decreasing systemic vascular resistance<sup>11</sup>. After the initial vasodilation, however, blood pressure may be restored to normal via reflex increases in heart rate and pulmonary arterial pressure<sup>1</sup>. Changes in ejection fraction have also been documented<sup>36</sup>, but these changes are primarily due to alterations in the function of the myocardium itself, specifically prolonged ventricular relaxation time resulting in increased left ventricular volume during diastole<sup>11</sup>. A 10% decrease in plasma volume has also been documented in horses receiving clenbuterol<sup>5</sup>, although the mechanism for this phenomenon is unknown.

### **2.6.2 Changes in Cardiac Dimensions**

Left ventricular hypertrophy is the most consistently reported effect of clenbuterol in humans and laboratory animals<sup>4,6,34,35</sup>, and increased mural thickness may be due to a combination of true hypertrophy<sup>34</sup> and myocardial fibrosis<sup>4</sup>. The dose of clenbuterol administered may play some role in the magnitude of changes detected; in 2 human trials with patients in cardiac failure, no echocardiographic changes were reported using a total clenbuterol dose of 80 $\mu$ g/day (approximately 0.5 $\mu$ g/kg q 12 h) for 12 weeks<sup>3</sup>, but an increase in end-diastolic left ventricular volume occurred when patients were administered clenbuterol at 720 $\mu$ g/day (approximately 3 $\mu$ g/kg q 8 h) for 12 weeks<sup>34</sup>. In horses receiving clenbuterol at 2.4 $\mu$ g/kg twice daily for 8 weeks, no echocardiographic alterations were evident at rest, but all parameters measured were significantly different in treated horses following strenuous exercise, except for end-diastolic thickness of the left ventricular free wall<sup>6</sup>.

### **2.6.3 Changes in Cardiac Function**

Besides inducing tachycardia, clenbuterol is also a positive inotrope and lusitrope. In rats, the prolonged diastolic relaxation induced by fenoterol (another  $\beta$  agonist) results in an increased left

ventricular filling pressure, which in turn reduces coronary blood flow and decreases cardiac output<sup>11</sup>. Conversely, post-exercise echocardiography in horses receiving clenbuterol documented an increase in stroke volume and cardiac output, as compared to control animals. However, the horses in this and other equine studies also demonstrated increased right ventricular pressures and impaired cardiac recovery post-exercise<sup>5,6</sup>. The conflicting conclusions regarding cardiac output may have been due to different measurement techniques.

The arrhythmogenic potential of clenbuterol has been documented in at least one human case of clenbuterol toxicity<sup>40</sup>. Proposed mechanisms for the increased risk of cardiac arrhythmia concentrate on dysregulation of intracellular calcium<sup>35</sup>. Both an increase in myofilament sensitivity to ionized calcium<sup>36</sup>, and an increase in the calcium content of the sarcoplasmic reticulum have been documented in animals administered clenbuterol, with prolongation of the action potential as a result<sup>33</sup>. Additionally, decreased taurine levels in cardiac tissue associated with clenbuterol administration may further destabilize cell membranes and contribute to intracellular calcium leakage<sup>51</sup>.

#### **2.6.4 Modulation of Cardiac Muscle Cell Death**

Similar to skeletal muscle, cardiomyocytes appear to exhibit a dose-dependent response to clenbuterol exposure. In the rat, apoptosis is first detected at clenbuterol doses of 1 µg/kg<sup>9</sup>, and necrosis occurs at doses ≥ 100 µg/kg<sup>12</sup>. Additionally, the primary site of clenbuterol-induced cardiac injury appears to be localized to a specific site near the apex of the left ventricle. The primary hypothesis behind this occurrence is that of relative cardiac hypoxia during exercise; as previously described, the reduction in coronary blood flow is greatest at this site. At this location, the maximum frequency of apoptosis was reported to be 0.3%<sup>9</sup>, and the maximum frequency of necrosis detected was 0.87-1.0%<sup>8,12</sup>. In cardiac muscle, stimulation of β<sub>1</sub> receptors may also contribute to cardiotoxicity, as both β<sub>1</sub> and β<sub>2</sub>-adrenoreceptor inhibition, as well as norepinephrine depletion, significantly reduced the myotoxic potential of clenbuterol in cardiac muscle<sup>9,28</sup>. Cardiotoxicity has been observed in humans<sup>40</sup> and horses at doses well below these levels.

Cardiac hypertrophy is reliably produced by clenbuterol administration; however some investigations found that a part of the increase in cardiac mass may be due to an increase in collagen mass, suggesting that fibrosis was due to irreversible induction of myocardial cell death. In one study,

clenbuterol induced a 12-15% increase in cardiac protein content, and a 3% increase in cardiac collagen content versus controls<sup>10</sup>. Further implications of these findings were discussed in the previous sections.

## **2.7 Ante-Mortem Evaluation of Myopathy in the Horse**

Several diagnostic modalities are available to the equine practitioner for detection of myopathy. Sufficiently severe myopathy of the skeletal musculature may result in widespread muscle necrosis or rhabdomyolysis, which is characterized by stiff and painful musculature and release of myoglobin. Common myopathies in Thoroughbreds and Quarter Horses include polysaccharide storage myopathy (PSSM) and recurrent exertional rhabdomyolysis (RER), both of which can contribute to poor performance and predispose the horse to clinical episodes of rhabdomyolysis. Cardiac dysfunction may also contribute to poor performance. Methods of evaluation for skeletal muscle injury and cardiomyopathy are outlined below.

### **2.7.1 Serum Biochemical Markers**

The most commonly used biochemical markers for evaluation of skeletal muscle injury in the horse are creatine kinase (CK) and aspartate aminotransferase (AST). Both are leakage enzymes that are released into circulation only when there is significant loss of cell membrane integrity, thereby making them useful in the diagnosis of muscle necrosis. Serum activity of CK peaks 4-6 hours following muscle injury. Due to its short half life, CK activity declines to normal levels within 24 hours in the absence of further muscle damage. Total CK activity is composed of 3 isoenzymes, CK-MM, CK-MB and CK-BB, which originate primarily from the skeletal muscle, cardiac muscle and brain, respectively. The skeletal muscle isoform contributes the majority of CK activity in the body; however, CK-MB constitutes 40% of cardiac CK activity and is not expressed in any other tissue.

By contrast, AST activity peaks 6-10 hours after muscle injury. The half-life of AST is 18 hours in the horse; therefore serum activity may take 4 or more days to return to normal levels. AST is also released from hepatocytes and osteocytes, and increases in AST enzyme activity must be interpreted carefully in light of CK activity and other organ function. Elevations in CK and AST activity in horses at rest suggest the presence of clinically significant myopathy. Post-exercise elevations in CK activity have also been used to support a diagnosis of subclinical myopathy in the evaluation of poor athletic performance; reported criteria include a three-to-four-fold elevation in CK activity 4 hours after



submaximal exercise<sup>52</sup>, or an increase in CK activity >1000U/L measured 30 minutes after a standardized exercise test<sup>53</sup>.

Cardiac troponin I (cTnI) is an alternative marker of myocardial injury that, similarly to CK, is also released from cardiac muscle during necrosis. Reference intervals (0-0.14ng/ml) have been established in diverse populations of healthy horses, including horses used for flat racing<sup>54-56</sup>. Significant elevations in cTnI activity in horses have been reported in cases of monensin cardiotoxicity<sup>57</sup>, valvular rupture<sup>58</sup>, clinically significant arrhythmias<sup>58,59</sup>, piroplasmosis<sup>60</sup> and other causes of myocarditis<sup>61,62</sup>. Measurement of cardiac troponin is considered highly reliable in human medicine for diagnosis of myocardial injury<sup>63</sup>, and clinical utility has also been documented in dogs<sup>64</sup> for diagnosis and monitoring of cardiomyopathy. Elevated cTnI was reported in one human case of clenbuterol overdose<sup>40</sup>.

### **2.7.2 Histopathology**

Histologic evaluation of a semimembranosus/semitendinosus muscle biopsy is the recommended procedure for identification of most myopathies that affect performance horses. Over 70% of Quarter Horses with a histologic diagnosis of PSSM carry a mutation on the GYS1 gene encoding for glycogen synthase<sup>65</sup>. This mutation has not been identified in Thoroughbreds affected with PSSM; therefore histologic examination remains the only available tool for diagnosis of this condition in Thoroughbreds. Detection of abnormal, amylase-resistant polysaccharide accumulation with periodic acid-Schiff stain is the hallmark feature of PSSM in a biopsy section<sup>66</sup>. Other myopathies may be identified by alterations in cellular morphology, such as loss of striations, atrophy, vacuolization or position of the nuclei within a cell, or by the presence of inflammatory cell infiltrate and/or fibrosis.

The mechanism of myocyte cell death may also be determined using immunohistochemical methods. In the mouse, Burniston *et al* describe the identification of skeletal and cardiac muscle necrosis using a staining technique for localization of anti-myosin antibody<sup>8,12</sup>. Briefly, anti-myosin antibody was administered intraperitoneally prior to induction of muscle injury, such that the anti-myosin antibody would bind to any myosin exposed during necrosis. This method does not translate well to use in a large animal such as the horse.

Apoptosis, or programmed cell death, is characterized histologically by irregular, pyknotic nuclei. It may be triggered through a multitude of enzymatic pathways, most (but not all) of which involve

activation of caspase 3. Another immunohistochemical target of apoptosis is the characteristic pattern of DNA digestion which produces multiple DNA fragments of a characteristic length, all possessing free 3'-OH termini. Common methods of identifying apoptosis in skeletal muscle *in situ* therefore rely on staining for caspase 3 or the 3'-OH DNA terminus. Both caspase 3 antibody and terminal deoxynucleotidyl transferase dUTP nick end labeling have been used successfully to demonstrate apoptosis in murine skeletal and cardiac muscle<sup>8-10,12</sup>, and they have also shown utility in identification of apoptotic cells in equine laminae<sup>67</sup> and intestine<sup>68</sup>.

### **2.7.3 Echocardiography**

Echocardiography permits direct sonographic visualization of the heart, allowing for the identification of physical abnormalities such as septal defects or transposition of the great vessels, valvular insufficiency or endocarditis, pericardial effusion or thickening, chamber enlargement, and alterations in contractility. Myocarditis may be difficult to appreciate sonographically unless marked fibrosis is present. Reference intervals for most echocardiographic measurements have been established for adult Thoroughbred horses<sup>69,70</sup>. A post-exercise echocardiogram is defined as a study performed during recovery from intense exercise, when the horse's heart rate exceeds 100 beats per minute<sup>53</sup>. Among Standardbred mares administered clenbuterol for 8 weeks, significant alterations in the post-exercise echocardiogram included increases in aortic root diameter, systolic thickness of the left ventricular free wall, thickness of the interventricular septum and left ventricular internal dimension<sup>6</sup>.

## CHAPTER 3. CLENBUTEROL TOXICITY IN 3 HORSES

### 3.1 Case Descriptions

#### 3.1.1 Horse 1

A 482 kg (1060 lb), 7-year-old Quarter Horse stallion (horse 1) from a racetrack in southern Louisiana was referred to the Veterinary Teaching Hospital & Clinics at Louisiana State University (LSU-VTH & C) with a 16-hour history of muscle fasciculation, hyperhidrosis, colic and stiff gait. Clinical signs occurred following oral administration of a compounded clenbuterol solution that was purported to contain 75µg/ml clenbuterol. The horse had been receiving clenbuterol as Ventipulmin® syrup (0.8µg/kg [0.36µg/lb] PO) once daily; however this was the first time the compounded product had been administered. Prior to referral, clinical signs had not responded to treatment with flunixin meglumine (1.1mg/kg [0.5mg/lb], IV, once), acepromazine and oral electrolyte solutions.

At presentation, muscle fasciculations were present over the entire body. The right gluteal and quadriceps muscles were firm and the horse was unwilling to bear full weight on the right hind limb. Lameness was not altered by perineural anesthesia of the right hind digital nerves at the level of the distal sesamoids. The horse was sweating, heart rate was increased (96 bpm) and capillary refill time was prolonged (5 seconds). Dehydration was estimated at 8% based on skin turgor. Nasogastric intubation recovered 7 L net reflux; however, rectal examination was unremarkable. Abdominal ultrasound revealed decreased corticomedullary definition and dilation of the renal pelvis in the right kidney.

Complete blood count showed hemoconcentration, leukocytosis and thrombocytopenia. Biochemical panel showed increased activities of CK and AST, azotemia, hyperbilirubinemia, hyperphosphatemia, hyperglycemia, hyponatremia and hypochloremia. Major clinicopathologic derangements at admission are summarized in Table 1. Urinalysis revealed isosthenuria, aciduria, glucosuria and a large amount of blood.

Emergency therapy consisted of methocarbamol<sup>a</sup> (12 mg/kg [5.45 mg/lb], IV, once), rapid infusion of intravenous crystalloid fluids<sup>b</sup>, insulin<sup>c</sup> (0.25U/kg [0.11U/lb], IM, once); unfractionated heparin<sup>d</sup> (40U/kg [20U/lb], SQ, q 8 h) and butorphanol<sup>e</sup> (0.04mg/kg, IV, once). Following the initial infusion, intravenous fluid therapy was continued at 2L/h with a balanced electrolyte solution supplemented with calcium borogluconate, potassium chloride and dimethyl sulfoxide. Despite transient improvement, the horse

became increasingly agitated and uncomfortable. Phenylbutazone<sup>f</sup> (4.4mg/kg [2mg/lb], IV, once), xylazine<sup>g</sup> (0.3mg/kg [0.15mg/lb], IV, once) and butorphanol (0.01mg/kg [0.005mg/lb], IV, once) failed to relieve signs of pain.

**Table 1.** Major laboratory abnormalities approximately 24 hours after clenbuterol overdose.

	Horse 1	Horse 2	Horse 3	Reference Interval
Hematocrit (%)	<b>59.9</b>	33.8	<b>41.5</b>	32-52
TP (g/dl)	<b>8.8</b>	6.1	<b>6.1</b>	6.1-8.1
Glucose (mg/dl)	<b>484</b>	461	<b>483</b>	70-105
BUN (mg/dl)	<b>32</b>	31	<b>42</b>	12-26
Creatinine (mg/dl)	<b>5.7</b>	3.5	<b>4.8</b>	1.2-2.0
CK (U/L)	<b>101,400</b>	6,970	<b>5,070</b>	0-350
AST (U/L)	**	**	<b>927</b>	0-350
ALP (U/L)	<b>331</b>	357	<b>614</b>	0-250
Ca (mg/dl)	<b>11.9</b>	9.4	<b>10.1</b>	11.3-13.4
Na (mmol/L)	<b>124</b>	135	<b>120</b>	130-140
K (mmol/L)	<b>3.4</b>	2.8	<b>3.0</b>	3.0-5.0
Cl (mmol/L)	<b>77</b>	93	<b>81</b>	97-105
PT (sec)	<b>13.2</b>	12.2	<b>12.6</b>	8-10
PTT (sec)	<b>60.6</b>	43.9	<b>44.9</b>	32-42
Band neutrophils (x10 <sup>3</sup> /μl)	<b>400</b>	0	<b>1,000</b>	
Platelets (x10 <sup>3</sup> /μl)	<b>92</b>	195	<b>118</b>	100-600

\*\* Not reported due to analyzer interference

Within 8 hours of admission, the horse became recumbent. Gluteal, quadriceps, and epaxial muscle groups were firm bilaterally, and whole body muscle tremors continued. Tachycardia (112 bpm) persisted, and a grade 3/6 end systolic heart murmur developed. Sinus tachycardia was confirmed with electrocardiography. Reevaluation of the serum biochemical panel revealed persistence of all previously described abnormalities except hyperglycemia and hyperphosphatemia. The leukogram on day 2 of hospitalization revealed development of a left shift.

Fluid therapy was amended to include endoserum<sup>h</sup> (500ml, IV, 0.5L/h) and hetastarch<sup>i</sup> in addition to crystalloid fluids. Intravenous calcium supplementation was also discontinued, and 7% sodium chloride (10ml/L) was added to the balanced electrolyte solution. Heparin therapy was discontinued, and naloxone<sup>j</sup> (0.016mg/kg [0.008mg/lb], IV, once), acepromazine<sup>k</sup> (0.06mg/kg [0.03mg/lb], IM, q 6 h), furosemide (0.5mg/kg [0.25lb], IV, once) and dexamethasone<sup>l</sup> (0.04mg/kg [0.02mg/lb], IV, once) were administered.

Analgesia was attempted with butorphanol (0.9mg/kg [0.4mg/lb], IM, once), xylazine (0.44mg/kg [0.2mg/lb], IV, once), detomidine<sup>m</sup> (0.2mg/kg [0.1mg/lb], IV, once), guaifenesin<sup>n</sup> (11.1mg/kg [5.0mg/lb], IV,

once). Constant rate infusion of butorphanol (0.013mg/kg/hr [0.006mg/lb/hr], IV) with periodic administration of diazepam<sup>o</sup> (0.1-0.2mg/kg [0.05-0.1mg/lb], IV, PRN) and detomidine provided adequate sedation to prevent self-mutilation. Additional supportive care included urinary catheterization, heavy bedding, manual repositioning, eye lubrication, and placement of protective headgear and lower limb bandages.

By the evening of day 2, the horse's condition had further deteriorated. Neurologic abnormalities included loss of coordinated motor activity in the rear limbs, absent menace responses, sluggish direct pupillary light reflexes, and development of nystagmus after repositioning. Tachycardia and sweating persisted, and body temperature decreased to 94.6°F (34.8°C). Disseminated intravascular coagulopathy was suspected based on thrombocytopenia and prolonged prothrombin (PT, 13.2 sec [8-10 sec]) and partial thromboplastin times (PTT, 60.6 sec [32-42 sec]). Azotemia and electrolyte derangements had not resolved despite aggressive fluid therapy.

The compounded product believed to have caused this patient's clinical signs was confirmed as pure clenbuterol by electrospray-tandem mass spectrometry. The concentration of clenbuterol in this product was determined to be 5.0mg/ml, which is approximately 70 times the concentration of the licensed product. Clenbuterol was also detected in the horse's serum at 5,450 pg/ml. Propranolol<sup>p</sup> (0.01mg/kg [0.005mg/lb]) was subsequently administered in 150ml physiologic saline solution as an intravenous infusion over 30 minutes. Following propranolol administration, heart rate slowed from 90 to 60 bpm, and the systolic heart murmur became less pronounced. Sweating and anxiety were subjectively decreased, but no effect on muscle stiffness was observed.

Dexamethasone (0.04mg/kg [0.02mg/lb], IV, once) administration was repeated, and omeprazole<sup>q</sup> (0.5mg/kg [0.23mg/lb], IV, q 24 h) and ceftiofur<sup>r</sup> (2.2mg/kg [1.0mg/lb], IM, q 12 h) were administered. Transdermal fentanyl patches<sup>s</sup> (0.67µg/kg/h [0.3µg/lb/h]) were applied. Diuresis with balanced polyionic solutions supplemented with magnesium sulfate (0.5g/L), potassium chloride (20mEq/L), and 7% sodium chloride (10ml/L) was maintained at a rate of 2L/h.

On day 3 of hospitalization, reevaluation of neurologic, renal and musculoskeletal abnormalities revealed further deterioration. The owners elected euthanasia, and the horse was euthanized by barbiturate overdose approximately 50 hours after clenbuterol ingestion.

### **3.1.2 Horse 2**

A 516 kg (1135 lb), 2-year-old racing Quarter Horse filly (horse 2) from the same racetrack as horse 1 presented approximately 24 hours after administration of the same compounded clenbuterol product. Excitation, hyperhidrosis, muscle tremors and tachycardia were observed shortly after administration. Treatments prior to admission included oral methocarbamol, milk of magnesia, valerian root, buttermilk, acepromazine (0.06mg/kg [0.03mg/lb], IM, once), dexamethasone (0.04mg/kg [0.02mg/lb], IV and IM, once each), flunixin meglumine (1.1mg/kg [0.5mg/lb], IV, once) and 8L crystalloid intravenous fluids.

Abnormalities at presentation included tachycardia (112 bpm), hyperhidrosis, muscle fasciculations and subcutaneous emphysema in the pectoral region. Electrocardiography confirmed sinus tachycardia. Central venous pressure (CVP), measured via the right jugular vein, was increased at 22 cmH<sub>2</sub>O.

Complete blood count was unremarkable. Serum biochemical analysis revealed hyperglycemia, azotemia, hyperbilirubinemia, hypocalcemia, hyperphosphatemia, hypochloremia and increased creatine kinase activity (Table 1). Serum clenbuterol concentration was confirmed to be 800 pg/ml by liquid chromatography-electrospray-tandem mass spectrometry.

A rapid intravenous infusion of 10L crystalloid fluids, supplemented with calcium borogluconate (4.6g/L), potassium chloride (20mEq/L), magnesium sulfate (1g/L) and dimethyl sulfoxide (13g/L), was administered, followed by 7% hypertonic saline (500ml, IV, once). Propranolol<sup>s</sup> (0.01mg/kg [0.05mg/lb], IV, once), diluted in 150ml physiologic saline solution, was administered over 30 minutes. In response to propranolol, heart rate decreased to 64 bpm, and CVP decreased to 15 cmH<sub>2</sub>O. Muscle tremors did not abate.

Butorphanol (0.03mg/kg [0.013mg/lb], IM, once), omeprazole (4.4mg/kg [2.0mg/lb], PO, q 24 h), and insulin (0.22U/kg [0.1U/lb], IM, once) were administered, and molded frog support cushions were applied on all four feet. Fluid therapy was maintained with Normosol-R at 1.5L/h, supplemented with calcium borogluconate (20ml/L), potassium chloride (20mEq/L) and magnesium sulfate (0.5g/L). Ceftiofur (2.2mg/kg [1.0mg/lb], IV, q 12 h) was also administered due to concerns about cellulitis in the pectoral region.

On day 2 of hospitalization, sinus tachycardia (64 bpm) and muscle fasciculations were still present. Transient fever (101.4°F [38.5°C]) was observed. Reevaluation of the serum biochemistry panel revealed resolution of hyperphosphatemia, hypocalcemia and hypochloremia, and improvement in the degrees of hyperglycemia, azotemia, hyperbilirubinemia, and CK elevation. Prothrombin time (12.2 sec) and partial thromboplastin time (43.9 sec) were mildly prolonged, but no clinical signs of coagulopathy were observed. Insulin (0.12U/kg [0.05U/lb], SQ, once) was repeated, and supportive care was continued.

By day 3 of hospitalization, blood glucose had normalized, azotemia was resolved, and heart rate returned to normal (40-48 bpm). No signs of laminitis developed despite the presence of mild digital pulses in the front feet. Intravenous fluid therapy was discontinued on the fourth day of hospitalization, and the filly was eating, drinking, defecating, and urinating appropriately. Serum biochemical profile on day 4 of hospitalization revealed residual elevations in total bilirubin and activities of CK, AST and ALP.

The filly was discharged after 5 days of hospitalization with no further medication required. All clinical signs and clinicopathological abnormalities had resolved at the time of release, except for mildly increased digital pulses.

### **3.1.3 Horse 3**

A 533-kg (1173 lb), 3 year old Quarter Horse gelding (horse 3) in race training and housed at the same barn as horses 1 and 2 was evaluated at the LSU-VTH & C for treatment of muscle tremors, profuse sweating, and tachycardia following oral administration of a compounded clenbuterol product 24 hours prior to admission. Flunixin meglumine (1.1mg/kg [0.5mg/lb], IV, twice), acepromazine (0.06mg/kg [0.03mg/lb], IM, once), dexamethasone (0.04mg/kg [0.02mg/lb], IV and IM, once each), lactated Ringer's solution (1L, IV, once) and oral methocarbamol were administered by the referring veterinarian. Valerian root and milk of magnesia were given by the horse's caretaker.

At presentation, sinus tachycardia (84 bpm), mild hypothermia (98.4°F [36.9°C]), profuse sweating, prolonged capillary refill time (3 seconds), and whole-body muscle fasciculations were detected. The quadriceps muscles were firm to the touch. Measurement of CVP was 7 cmH<sub>2</sub>O via the right jugular vein. Initial fluid therapy consisted of rapid intravenous administration of both crystalloid and

colloid fluids (Normosol-R, 10L, supplemented with calcium borogluconate at 4.6g/L, potassium chloride at 20mEq/L and magnesium sulfate at 1mg/L; hetastarch 6%, 2L; 7% hypertonic saline, 500ml).

Hyperglycemia, azotemia, hyperbilirubinemia, hyperphosphatemia, hypochloremia, and increased activities of CK, AST and ALP were present. A complete blood count was unremarkable. Urinalysis revealed isothermia, glucosuria, a large amount of blood, and trace proteinuria and ketonuria.

Following fluid resuscitation, CVP increased to 26 cmH<sub>2</sub>O, and the horse became more agitated and tachycardic. Propranolol (0.01mg/kg [0.005mg/lb]), diluted in 150ml physiologic saline solution, was administered intravenously over 30 minutes. Heart rate, CVP and anxiety decreased following propranolol administration, but muscle tremors and sweating continued. Insulin (0.22U/kg [0.1U/lb], IM, once), butorphanol (0.028mg/kg [0.013mg/lb], IM, q 8 h), heparin (19U/kg [8U/lb], SQ, q 6 h) and omeprazole (1.1mg/kg [0.5mg/lb], PO, q 24 h) were administered, and frog support pads were applied to the front feet. Fluid therapy with balanced polyionic fluids was maintained at 1.5L/hr (Normosol-R, supplemented with calcium borogluconate (4.6g/L), potassium chloride (20mEq/L), magnesium sulfate (0.5g/L), and dimethyl sulfoxide (13g/L).

On day 2 of hospitalization, tachycardia (76 bpm) and muscle tremors were still present, and mild pyrexia (101.5°F [38.6°C]) developed. Reevaluation of clinicopathologic parameters revealed resolution of hyperphosphatemia. Hyperglycemia, azotemia, hypochloremia, and increased CK activity persisted, and activities of AST and ALP had increased since admission. A left shift (1,000 band neutrophils/μl) was evident on the complete blood count, despite a total leukocyte count (5,600/μl) and absolute neutrophil count (3,200/μl) within the reference interval. Clotting times (PT, 12.6 sec; PTT, 44.9 sec) were mildly prolonged. Propranolol administration was repeated as previously described and successfully decreased the heart rate by about 10 bpm. Insulin (19U/kg [8U/lb], SQ, once) was also repeated, and all other therapy was continued.

The horse subsequently developed mild bilateral forelimb lameness that progressed to signs of acute laminitis by the end of day 2, despite application of ice to the front feet. Tripellenamine<sup>t</sup> (0.75mg/kg [0.34mg/lb], IM, once), flunixin meglumine<sup>u</sup> (0.5mg/kg [0.22mg/lb], IV, once) and pentoxifylline<sup>v</sup> (7mg/kg [3.2mg/lb], diluted in 1L 0.9% saline, IV, q 8 h) were given. Reassessment of the serum biochemistry panel revealed resolution of hyperglycemia and decreasing azotemia.



The third day of hospitalization, the horse showed signs of acute laminitis in all 4 feet, and muscle tremors persisted. No displacement of the distal phalanx was evident on lateral and dorsopalmar radiographs of all 4 feet. Rectal temperature (101.5°F [38.6°C]), pulse (88 bpm), and respiratory rate (52 bpm) were all elevated. Perineural anesthesia of the digital nerves of all 4 limbs produced a temporary but significant decrease in heart rate. Serum biochemical abnormalities were resolved, with the exception of azotemia (BUN 27mg/dl, creatinine 2.7mg/dl) and elevated activities of AST (1060 U/L), ALP (959 U/L) and CK (1550 U/L). Mild leukopenia (4,500/μl) and neutropenia (1,800/μl) developed, and a persistent left shift (200 bands/μl) was present.

Phenylbutazone (4.4mg/kg [2mg/lb], IV, q 24 h) and butorphanol (0.056mg/kg [0.025mg/lb], IM, once) were administered. Frog supports, lower limb support bandages and ice were applied to all 4 limbs. Under heavy sedation, the horse was placed in a sling. Muscle fasciculations ceased when the horse was non-weight bearing. A constant rate infusion of ketamine<sup>w</sup> (0.4mg/kg/h [0.2mg/lb/h], IV) was initiated while the horse was sedated and continued while slung. Crystalloid fluids and other medications were continued. Feces became soft on day 3; di-trioctahedral smectite<sup>x</sup> was administered.

By the morning of day 4 of hospitalization, the patient was still tachycardic (80 bpm). Complete blood count revealed mild neutropenia (1900/μl) with a degenerative left shift (1300/μl), lymphopenia (800/μl), and monocytosis (1100/μl). Elevations in serum activities of AST (1179 U/L), ALP (1108 U/L) and CK (1408 U/L), hypocalcemia (9.8mg/dl), hyperbilirubinemia (4.9mg/dl) and hypoproteinemia (5.3g/dl) were detected. Over the next 12 hours, fecal consistency returned to normal, and smectite treatment was discontinued, along with ice application and heparin. Fluid administration rate was reduced to 1L/h, and the gelding was removed from the sling. Muscle fasciculations, tachycardia (84 bpm) and tachypnea (72 bpm) recurred after removal from the sling. Oral mucous membranes also became congested, and a fever of 102.1°F (38.9°C) developed. Endoserum (1 L, IV at 0.5L/h) was administered but did not alter vital parameters.

On the 5<sup>th</sup> day of hospitalization, the patient was febrile (102.4°F [39.1°C]), tachycardic (72 bpm) and severely tachypneic (84 bpm), with red, injected mucous membranes and a capillary refill time of 3.5 seconds. The horse continued to exhibit signs of laminitis. Unilateral serosanguinous discharge was also noted from the left nostril. Thoracic ultrasound findings were consistent with pleuropneumonia. Comet

tail artifacts were diffusely distributed throughout the cranial lung fields bilaterally, and bilateral pulmonary consolidation was evident cranioventrally. Bilateral, anechoic pleural effusion ( $\leq 1$  cm) was present cranioventrally, and pericardial effusion was also identified.

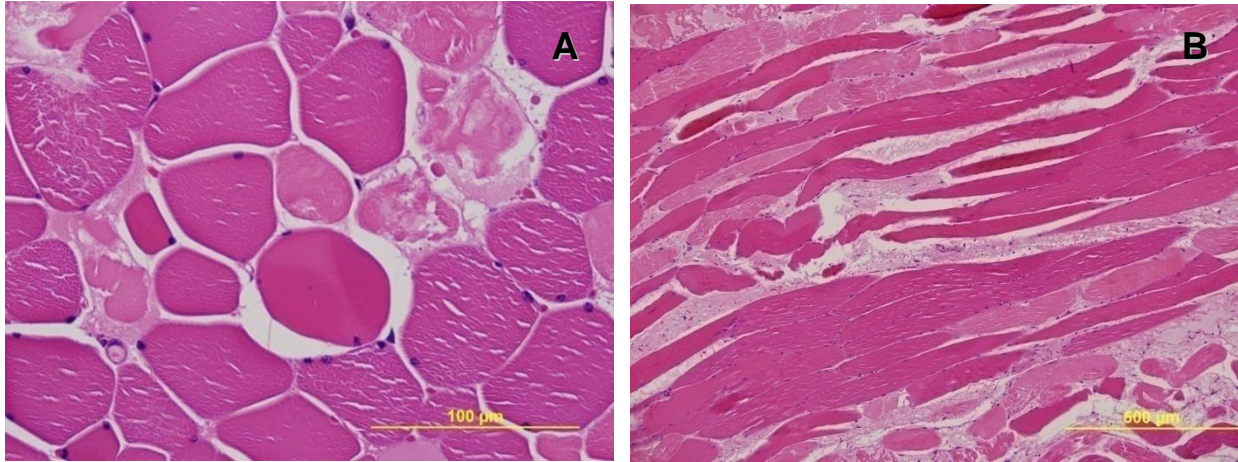
Clinicopathologic reevaluation revealed hyperbilirubinemia (6.8mg/dl), persistently elevated AST (1128 U/L) and ALP (1421 U/L) activities, hypophosphatemia (2.1mg/dl), and hypoproteinemia (5.1g/dl). Creatine kinase activity had decreased to nearly normal levels (363 U/L, reference 0-350 U/L). Hyperfibrinogenemia (700mg/dl) and neutrophilic leukocytosis (16,500 WBC/ $\mu$ l, 11,200 neutrophils/ $\mu$ l) with a left shift (3,600 bands/ $\mu$ l) were present. Humane euthanasia was performed at the owner's request. The horse was euthanized by barbiturate overdose.

### **3.2 Post-Mortem Findings**

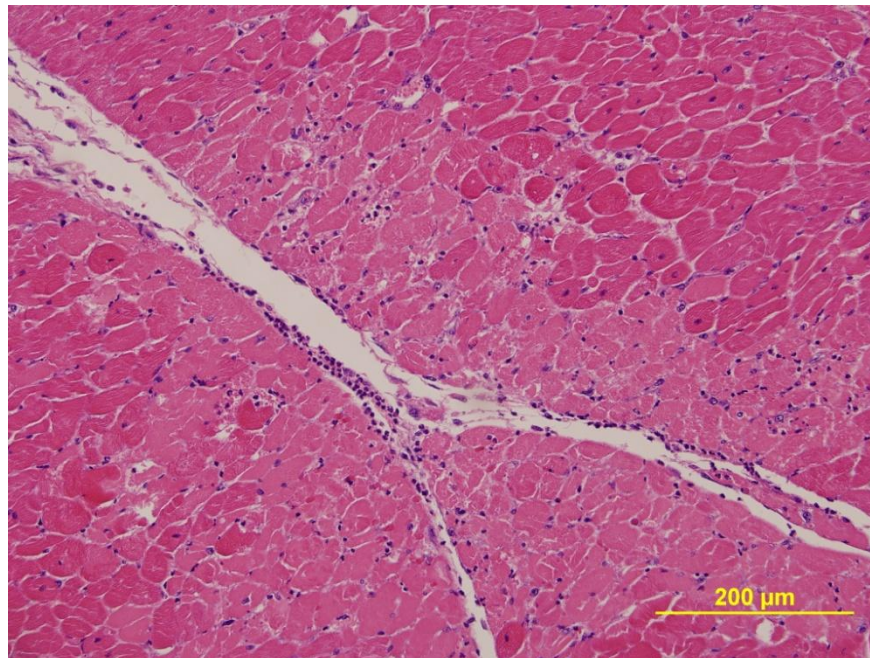
#### **3.2.1 Horse 1**

The muscles of the upper pelvic limbs contained multifocal to coalescing areas of abnormally firm, pale pink to white discoloration bilaterally. Histologically, these muscles displayed multifocal to coalescing necrosis of myocytes, affecting individual fibers as well as large muscle bundles. Skeletal myocyte necrosis was characterized by hypereosinophilia, cytoplasmic vacuolization, loss of cellular detail, swollen myofibers, and loss of cross-striations. Hypaxial muscles were diffusely swollen, soft, and were mottled with dark red, patchy to coalescing areas of hemorrhage. Myocyte necrosis, characterized by hypereosinophilia, fragmentation of muscle fibers, loss of cellular detail, loss of cross-striations, and muscle fiber loss, was evident histologically (Figure 2). Frequent blood clots, defined by large areas of hemorrhage containing fibrin and inflammatory cells, were also interspersed in the hypaxial muscles.

Although not grossly abnormal, the myocardium was necrotic on histologic examination. Degenerate to necrotic cardiomyocytes occurred as scattered individual cells and in small clusters, characterized by myofiber fragmentation, hypereosinophilia, cytoplasmic granularity, and occasional loss of cross-striations (Figure 3). Multifocal interstitial edema and focal subendocardial hemorrhage were present.



**Figure 2.** Skeletal muscle from horse 1, hematoxylin & eosin. Cross-sectional (A, 600X) and longitudinal (B, 100X) photomicrographs demonstrate extensive cellular injury via Zenker's necrosis, contraction band formation and myocyte fragmentation.



**Figure 3.** Cardiac muscle from horse 1, hematoxylin & eosin, 200X. A cluster of hyper-eosinophilic, necrotic cardiac myocytes is noted in the center of this photomicrograph.

The bladder contained reddish-brown, cloudy urine. Mild to moderate renal tubular degeneration and necrosis was present in the renal cortex, characterized by individual hyper-eosinophilic cells with loss of nuclear detail, sloughed pyknotic cells within the tubular lumen, or the occasional presence of flattened epithelial cells lining renal tubules. In the tubules of the cortex and medulla, accumulation of eosinophilic, proteinaceous globules and red, granular casts was widespread. Together with the post-mortem

confirmation of rhabdomyolysis, kidney lesions were consistent with acute renal failure due to pigment nephropathy.

Moderate, diffuse, hepatic hemosiderosis, and pulmonary congestion and edema with severe hemosiderosis were also present. Changes in the lungs, liver, and heart were compatible with acute heart failure or acute cardiac insufficiency.

The samples of liver and kidney obtained post-mortem, approximately 48 hours post-clenbuterol administration, as well as the serum sample drawn 24 hours post-administration, were analyzed for clenbuterol residues by liquid chromatography-electrospray-tandem mass spectrometry. All samples were positive for clenbuterol, with tissue concentrations being significantly higher than serum concentration. Clenbuterol in serum 24 hours after ingestion was present at 5,450pg/ml. At 48 hours after ingestion, clenbuterol was detected in liver at 376,890pg/ml and in kidney at 202,240pg/ml.

### **3.2.2 Horse 3**

Skeletal muscle lesions in horse 3 were limited to the detection of diffuse, small, multifocal areas of myocyte mineralization. The myocardium of the ventricular free walls and interventricular septum was diffusely and asymmetrically discolored by pale tan streaking, with the left ventricular free wall being the most severely affected. Approximately 40% of the heart exhibited tan discoloration grossly, and 25% of sections examined microscopically exhibited cardiomyocyte necrosis or inflammatory changes. Cardiomyocyte necrosis was characterized by loss of cross-striations, fragmentation, cellular vacuolization, karyolysis, nuclear pyknosis, and sarcolemmal sheath collapse. Perivascular tissue and areas of the myocardium surrounding Purkinje cells were expanded with granulomatous infiltrate and either non-staining fluid or a basophilic, mucinous material. Approximately 200 ml of dark yellow fluid was present in the pericardial sac. Aerobic bacterial culture of pericardial fluid was negative.

Subcapsular petechial hemorrhage was observed in the kidneys bilaterally. Non-specific degenerate and necrotic lesions were identified in the renal tubules, along with evidence of tubular epithelial repair. The bladder contained normal-appearing urine.

The cranioventral lung was sharply demarcated bilaterally by dark green consolidation, encompassing about 20% of the lung parenchyma, and the remaining lung tissue was rubbery and partially collapsed. Interlobular spaces in the consolidated area were expanded with edema and yellow,

friable material consistent with fibrin, and the pleural surface was covered by a thin layer of similar brown, friable material. The trachea and bronchii were filled with clear to white foam. Aerobic bacterial culture of affected lung yielded a mixed population of *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp. and *Proteus* spp., consistent with aspiration pneumonia. Histologically, parenchymal necrosis, edema, vascular necrosis and suppurative exudate were evident in the cranioventral lungs, and large numbers of chain-forming cocci were present in necrotic foci. Neutrophilic infiltration was identified in the adventia and mucosa of the trachea.

The liver was firm with an accentuated lobular pattern on gross examination. Mild vacuolization of centrilobular hepatocytes, mild biliary hyperplasia and mild lymphocytic infiltration of the portal triads were present.

The glandular portion of the stomach was thickened and hyperemic, and about half of the affected area was covered with a fibrinous pseudomembrane that was colonized with coccobacilli. Mild lymphocytic-plasmocytic inflammation was also present in the small intestine and large colon. Laminar edema of the right fore and left rear feet was noted, consistent with laminitis.

The tissue samples and stomach contents obtained post-mortem, approximately 114 hours post-clenbuterol administration, as well as the serum sample drawn 24 hours post-administration, were analyzed for clenbuterol residues by liquid chromatography-electrospray-tandem mass spectrometry. All samples were positive for clenbuterol. Serum clenbuterol concentration 24 hours post-ingestion was 1,050pg/ml. At 114 hours post-ingestion, clenbuterol was detected in liver at 6,790pg/ml, in kidney at 1,700pg/ml, in spleen at 1,550pg/ml, in brain at 1,080pg/ml, and in stomach contents at 1,150pg/ml.

### **3.3 Discussion**

This report describes 3 cases of severe clenbuterol overdose in the horse. All horses exhibited prolonged sinus tachycardia, muscle tremors, hyperhidrosis and anxiety. Similarly, reports of human clenbuterol toxicity cite nervousness (91%), tachycardia (82-91%), distal tremors (75-88%), nausea (74%), epigastralgiias (68%), headaches and dizziness (36-42%), fever (36%), myalgias (20-22%), hypertension (18%), and vomiting (18%) as the most common symptoms<sup>21,71</sup>. Horse 1 was admitted for a history of colic signs, consistent with the high frequency of gastrointestinal upset in people. In our equine patients, fever was an inconsistent finding. Horse 1 remained normothermic to hypothermic, possibly due

to derangements in tissue perfusion. A single occurrence of fever was observed in horse 2 on the second day of hospitalization and resolved spontaneously. The mild pyrexia observed in horse 3 was initially attributed to clenbuterol effects, but its persistence may have been a sign of developing pneumonia.

The horses in this report were estimated to have received 10µg/kg (horses 2 and 3) or 100µg/kg (horse 1) of the compounded clenbuterol. For treatment of respiratory disease in horses, clenbuterol is approved for use at doses up to 3.2µg/kg. The serum concentration of clenbuterol detected in horse 1 at admission was approximately 25 times higher than expected for a horse given clenbuterol within the labeled dose range, based on a 24-hour withdrawal time<sup>25</sup>.

In the horse, side effects at or below 3.2µg/kg include mild tachycardia, anxiety, muscle tremors and sweating, but signs are reported to subside within an hour of administration<sup>1</sup>. Conversely, symptoms of clenbuterol toxicity in humans may last up to 6 days, and complications such as seizures and supraventricular arrhythmias have been reported<sup>20,21,37,38,40</sup>, more closely resembling the cases described in this report. Estimates of oral clenbuterol doses resulting in human toxicosis range from 0.9µg/kg<sup>20</sup> to 4.8mg/kg<sup>37</sup>. In one human case report, clinical signs persisted even after serum concentrations of clenbuterol had decreased below detectable levels<sup>38</sup>. A similar phenomenon was present in our equine patients.

Documentation of elevated tissue concentrations of clenbuterol in horses 1 and 3 may explain the prolonged effects of clenbuterol (Table 2). Tissue clearance of clenbuterol is often prolonged relative to serum clearance, and prolonged detection of clenbuterol is reported in the liver, kidney, eye fluids, lung, heart and spleen of horses<sup>26</sup>. Due to its metabolism in the liver and excretion in the urine<sup>25</sup>, the liver and kidney frequently contain concentrations of clenbuterol several times higher than that of serum<sup>20,21</sup>.

**Table 2.** Serum and selected tissue clenbuterol concentrations after clenbuterol overdose (ng/ml).

Time Post-Admin.	Horse 1		Horse 2	Horse 3	
	24 h	50 h	24 h	24 h	114 h
Serum	5.45		0.80	1.05	
Liver		376.89			6.79
Kidney		202.24			1.70
Stomach Contents					1.15
Brain					1.08
Spleen					1.55

Although the clinical signs of clenbuterol toxicity in horses closely parallel those in the human literature, some of the clinicopathologic abnormalities appear to be unique. In people, hypokalemia, hyperglycemia, hypomagnesemia and neutrophilic leukocytosis are frequently reported<sup>20,22,38</sup>. Hypokalemia in all 3 horses was mild to absent. Serum magnesium concentrations were not measured in our patients, but supplemental magnesium was provided based on human reports of hypomagnesemia with clenbuterol overdose. Immature (band) neutrophils were detected in both non-surviving horses, but only horse 1 exhibited absolute neutrophilia at admission.

The predominant alteration in the serum chemistry profile of all 3 horses was profound hyperglycemia (Table 1). Hyperglycemia results from induction of glycogenolysis by hepatic  $\beta_2$  adrenoreceptors. This effect may be compounded by prolonged exposure of hepatic tissues to clenbuterol; not only is the drug metabolized in the liver, but hepatic concentrations far exceed serum levels for several days<sup>26</sup>. In contrast to the human literature, hyperglycemia in our equine patients did not resolve spontaneously, and repeated administration of insulin was required to achieve normoglycemia. Severe hyponatremia and hypochloremia are not reported in humans, but both abnormalities were associated with non-survival in horses 1 and 3.

Adverse effects of clenbuterol may be broadly considered to arise from excessive adrenergic stimulation. Clenbuterol is a moderately selective  $\beta_2$  adrenergic agonist, but at higher doses, all classes of  $\beta$ -adrenoreceptors are activated.  $\beta_2$  adrenergic receptors are present in equine skeletal and cardiac myocytes, hepatocytes, nervous tissue, respiratory smooth muscle, vascular smooth muscle, uterus, and ileum<sup>1,12,27-30</sup>. In the vasculature, therapeutic doses of clenbuterol initially produce a decrease in blood pressure, but this is counteracted within 2 minutes of administration by a reflex increase in heart rate and pulmonary arterial pressure<sup>1</sup>. The higher doses of clenbuterol administered to our patients may have contributed to the profound hypotension and alterations in perfusion seen in the 2 more severely affected horses; possibly contributing to the development of laminitis in horse 3 and complicating treatment of rhabdomyolysis in horse 1, respectively.

$\beta_1$  adrenergic receptors in the myocardium may be stimulated directly by clenbuterol, or secondarily by norepinephrine released from presynaptic sympathetic neurons responding to  $\beta_2$  activation<sup>28</sup>. In both skeletal and cardiac muscle, however, direct toxicity is mediated through pathways

downstream of the  $\beta_2$  receptor. In rats, a single administration of clenbuterol at or above 10 $\mu$ g/kg produces skeletal muscle necrosis, and doses at or above 100 $\mu$ g/kg induce cardiac necrosis<sup>8,10,12</sup>. In the heart, this effect is most pronounced in the left ventricle. With repeated administration at or below 10 $\mu$ g/kg in rats, clenbuterol appears to induce muscle hypertrophy without adverse side effects; the attenuation of myotoxic effects over time is thought to be related to downregulation of adrenoreceptors<sup>10</sup>. Skeletal and cardiac muscle injury was demonstrated in both non-surviving horses. The dose of clenbuterol administered to horse 3 was well below the dose which is reported to induce cardiac necrosis in rats.

Clenbuterol also activates  $\beta_3$  (or “ $\beta$ -atypical”) receptors, which are purportedly responsible for the lipolytic effects of clenbuterol, and which are relatively insensitive to blockade by the majority of  $\beta$ -adrenergic antagonists, including propranolol<sup>1,27</sup>.  $\beta_3$  receptors have been identified in vascular smooth muscle, adipocytes, liver, and the equine ileum. Activation of these receptors may contribute to the poor response to  $\beta$ -blocker therapy in some patients.

A variety of targeted and symptomatic therapies have been used in clinical cases of human clenbuterol ingestion, including  $\beta$ -blockers, anti-emetics, potassium chloride, and anti-convulsants such as diazepam<sup>71</sup>. The  $\beta$ -blockers, including propranolol, are the most pharmacologically direct means of counteracting  $\beta$ -adrenergic stimulation. Together with intravenous potassium chloride, they are considered the treatment of choice for acute clenbuterol exposure in human medicine<sup>41,71</sup>. Repeated administration of  $\beta$ -blockers may be required to control tachycardia and other manifestations of toxicosis in people<sup>38,40</sup>. A similar phenomenon was observed in our equine patients and was attributed to accumulation of clenbuterol in tissues or contributions from (refractory)  $\beta_3$  adrenoreceptor activity.

Although propranolol was effective at reducing heart rate in all cases, neither propranolol, any sedative agent, nor any of the analgesics administered were effective at reducing anxiety and muscle tremors. Provision of analgesia using a constant rate infusion of ketamine in the conscious horse has been previously described<sup>72</sup>. Administration of ketamine using this protocol was attempted in horse 3 following the onset of laminitis. Although no adverse effects on mentation were noted, the desired analgesic effects were likewise not appreciated in this case.



Interestingly, all of our patients had received dexamethasone for symptomatic treatment prior to admission. With chronic clenbuterol administration, tolerance develops as  $\beta_2$  receptors are downregulated in lymphocytes, smooth muscle, and skeletal muscle, but receptor tachyphylaxis is reversible by the administration of dexamethasone<sup>46</sup>. Although all horses presented with acute clenbuterol overdose were accustomed to receiving clenbuterol, the protective effects afforded by previous downregulation of  $\beta$ -adrenoreceptors could have been removed by dexamethasone-induced upregulation shortly after the overdose.

In rats, pretreatment with selective  $\beta_1$  adrenergic antagonists,  $\beta_2$  adrenergic antagonists and norepinephrine depletion with reserpine all attenuated cardiomyocyte injury by 91-100% in the face of clenbuterol administration. However, treatment with  $\beta_1$  antagonists and reserpine did not reduce skeletal myocyte necrosis<sup>12,28</sup>. Reserpine has been used to achieve long-term behavioral modification in horses. Based on the aforementioned studies in rats, this drug may also have the potential to attenuate cardiotoxicity in horses if given immediately after intoxication, or it may reduce the need for repeated  $\beta$ -blocker administration to control tachycardia.

Enhancement of lean muscle mass and performance are the primary reasons for clenbuterol abuse. However, several scientific investigations question the efficacy of clenbuterol as a performance-enhancing agent<sup>4-7,12,48</sup>. While clenbuterol has been repeatedly demonstrated to increase overall skeletal and cardiac muscle mass<sup>3,10,33,34,44</sup>, its effects on tensile strength are variable<sup>3,34,48</sup>. Decreases in exercise performance and an increased incidence of sudden cardiac failure have been described in rats<sup>4</sup>.

Studies investigating the effects of clenbuterol in both exercising and non-exercising horses demonstrated repartitioning effects<sup>44,47</sup>, changes in myosin heavy chain composition<sup>45</sup>, cardiac function<sup>6</sup>, and aerobic exercise performance<sup>5,7</sup> using doses of clenbuterol within the recommended therapeutic range for respiratory disorders. Demonstration of these same effects in rat models required doses up to 100 times higher than those used to treat respiratory disease in horses. These findings suggest that horses may possess a different threshold for  $\beta$ -adrenergic agonist-mediated skeletal and cardiac muscle response and injury than laboratory animal species. Closer investigation into the potential risks of clenbuterol use among equine athletes not suffering from restrictive airway conditions is warranted.

In summary, a compounded formulation of clenbuterol was shown to be responsible for the severe and prolonged clinical signs described in these 3 cases. A three- to 30-fold overdose was suspected. The drug was present in the serum and tissues of these horses in excess of concentrations expected from routine administration of the medication. Major features of toxicity included prolonged sinus tachycardia, muscle tremors, hyperhidrosis and hyperglycemia. Complications were severe and included rhabdomyolysis, renal failure, cardiotoxicity and laminitis. Propranolol was successful in reducing heart rate in all horses, but it did not alleviate other signs of toxicity. The horse with the lowest serum concentration of clenbuterol at admission survived. The high mortality rate of clenbuterol toxicosis compared to human reports is attributable to the magnitude of the overdose in these horses.

### **3.4 Product Information**

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<sup>a</sup> Robaxin®-V, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>b</sup> Normosol®-R, Abbott Animal Health, Abbott Park, IL, USA

<sup>c</sup> Vetsulin®, Intervet/Schering-Plough Animal Health, USA

<sup>d</sup> Heparin Sodium Injection (10,000U/ml), USP, Abraxis Pharmaceutical Products, Schaumburg, IL, USA

<sup>e</sup> Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>f</sup> Butaject 20%, Butler Animal Health Supply, Dublin, OH, USA

<sup>g</sup> AnaSed® Injection, Lloyd Laboratories, Shenandoah, IA, USA

<sup>h</sup> Endoserum®, IMMVAC, Inc., Columbia, MO, USA

<sup>i</sup> 6% Hetastarch, Baxter Healthcare Corporation, Deerfield, IL, USA

<sup>j</sup> Naloxone Hydrochloride Injection (0.4mg/ml), USP, Hospira, Lake Forest, IL

<sup>k</sup> PromAce® Injectable, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>l</sup> Dexamethasone Sodium Phosphate, 4mg/ml, USP, APP Pharmaceuticals, Schaumburg, IL, USA

<sup>m</sup> Dormosedan®, Pfizer Animal Health, Exton, PA, USA

<sup>n</sup> Guaifenesin (in 5% dextrose) for IV injection, 50mg/ml, Wedgewood Pharmacy, Swedesboro, NJ, USA

<sup>o</sup> Diazepam Injection (5mg/ml), USP, Hospira, Lake Forest, IL, USA

<sup>p</sup> Propranolol (1mg/ml), USP, Baxter Healthcare Corporation, Deerfield, IL, USA

<sup>q</sup> Omeprazole Injection, 75mg/ml, Premier Pharmacy Labs, Inc., Weeki Wachee, FL, USA

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<sup>r</sup> Naxcel®, Pfizer Animal Health, Exton, PA, USA

<sup>s</sup> Duragesic® 100mcg/hr, Ortho-McNeil-Janssen Pharmaceuticals, Inc., Raritan, NJ, USA

<sup>t</sup> Re-Covr® Injection, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>u</sup> FlunixiJect, Butler Animal Health Supply, Dublin, OH, USA

<sup>v</sup> Pentoxifylline for IV injection, 4g, HDM Pharmacy, LLC, Lexington, KY, USA

<sup>w</sup> Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>x</sup> BioSponge™, Platinum Performance, Buellton, CA, USA

## CHAPTER 4. EXPERIMENTAL INVESTIGATION OF THE EFFECTS OF CLENBUTEROL ON SKELETAL AND CARDIAC MUSCLE

### 4.1 Introduction

Clenbuterol belongs to a class of bronchodilator agents known as  $\beta_2$  agonists. It is a moderately selective  $\beta_2$ -adrenoreceptor agonist that is licensed in the United States for the treatment of horses with obstructive lower airway disease. Due to the multisystemic distribution of  $\beta_2$  receptors within the body, clenbuterol also produces extra-therapeutic effects, both desirable and detrimental. Its augmentation of lean muscle mass has made illegal use popular among livestock producers and human bodybuilders, and its use is tightly regulated in performance horses, where the potential for abuse is also perceived to be high. Cases of human clenbuterol toxicity have been reported with direct ingestion<sup>37,38,40</sup> and secondary to consumption of clenbuterol-contaminated meat products<sup>20-23</sup>. Side effects in humans range from transient muscle tremors and gastrointestinal upset to severe, prolonged tachycardia with muscle tremors, electrolyte derangements and supraventricular arrhythmias. Three cases of clenbuterol toxicity in horses were reported in the previous chapter, in which clinical signs resembled those reported in severely affected human beings.

In rats, clenbuterol reliably induces either necrosis or apoptosis in skeletal and cardiac muscle, based on the dose administered<sup>8-12</sup>. Generally speaking, high doses induce necrosis, and lower doses induce apoptosis. However, both equine and human cases of toxicity have been reported after ingestion of clenbuterol doses well below those which cause signs of toxicity or measurable muscle damage in murine species. These studies suggest that horses and humans may be more sensitive to  $\beta$ -adrenergic stimulation than rodents.

Previous studies have suggested that clenbuterol has a negative effect on aerobic athletic performance<sup>7</sup>, but a specific mechanism for this effect has not been proven. Some previously reported effects of clenbuterol, which may explain a reduction in aerobic capacity, include direct  $\beta_2$ -mediated myotoxicity resulting in apoptotic or necrotic cell death<sup>8-10,12,28</sup>, reduction of intracellular myocyte phosphofructokinase activity<sup>42</sup>, and increased frequency of type IIX (fast-twitch) muscle fibers in predominantly slow-twitch muscles<sup>42,45,49</sup>. The mechanism by which this change in predominant myosin heavy chain type occurs is unknown. Based on documentation of skeletal muscle damage in the cases of clenbuterol toxicity reported in the previous chapter, one may hypothesize that changes occur because of

or in association with subclinical muscle damage in horses receiving “therapeutic” doses of clenbuterol. No studies to date have examined the effects of exercise and clenbuterol on equine skeletal muscle morphology or frequency of apoptosis in the horse, nor has alteration in echocardiographic function been evaluated in horses receiving the dosing regimen employed in this study.

The purpose of the study described herein was to determine if evidence of skeletal and/or cardiac muscle damage could be detected in horses receiving oral clenbuterol at doses commonly prescribed for respiratory disease. Additionally, this study sought to quantify and compare echocardiographic measurements, serum muscle enzyme activities and histologic characteristics of skeletal muscle in horses receiving clenbuterol versus saline-treated control horses.

## **4.2 Materials and Methods**

Twenty-three Thoroughbred mares and geldings were evaluated for inclusion in this study. All animals were obtained from the research herd maintained by the Louisiana State University Equine Health Studies Program and were returned to the herd at the conclusion of the study. This study conformed to the University guidelines for animal use and was approved by the Louisiana State University Institutional Animal Care and Use Committee.

### **4.2.1 Animal Selection and Treatment Group Assignment**

Horses were between 3 and 10 years old at the time of evaluation and were free of obvious lameness (less than grade 1/5 on all limbs, using the AAEP grading system). Animals were determined to be healthy based on routine physical examination, complete blood count and serum biochemistry panel.

An echocardiogram was performed during initial evaluation. Horses were excluded if significant alterations in echocardiographic measurements of left ventricular size or function were detected<sup>69,70</sup>. Thoracic auscultation during rebreathing and endoscopic examination of the upper airway and trachea were performed. Horses were excluded if abnormal lung sounds were detected on auscultation or the tracheal mucus score was greater than 1 on endoscopic examination<sup>18</sup>. Histopathology of semimembranosus muscle biopsies was performed at an outside laboratory<sup>a</sup>, and horses were excluded if significant lesions were reported.

Twelve horses met all selection criteria and were randomly assigned to either the control group (n=6) or the clenbuterol group (n=6).

#### **4.2.2 Study Overview**

Horses were housed in individual box stalls for 48 hours after initial evaluation for observation of pain or other adverse events associated with the biopsy site. Pelleted feed<sup>b</sup> was provided twice daily, and horses had constant access to water while stalled. A physical examination was performed once daily. Horses were then turned out on pasture for at least 12 days before beginning the treatment protocol.

After a minimum of 14 days from initial evaluation, the treatment protocol was initiated. Horses were housed in bedded box stalls during this time, and twice daily pelleted feed<sup>b</sup> and once daily grass hay were provided. Water was available ad libitum. A physical examination was performed once daily, and horses were monitored for signs of clenbuterol toxicity, including muscle fasciculation or stiffness, anxiety, tachycardia and excessive sweating.

Horses were subjected to daily submaximal treadmill exercise during the treatment period. Venous blood was collected from the jugular vein into sterile evacuated tubes containing no additives<sup>c</sup>, sterile evacuated tubes containing lithium heparin<sup>d</sup> and sterile evacuated tubes containing K<sub>2</sub>EDTA<sup>e</sup> during initial evaluation (pre-treatment, day 0) and on days 3, 6, 9 and 12 of treatment. During the treatment period, samples were taken immediately prior to morning treatment administration, which was approximately 12 hours after the previous evening's treatment. Serum was separated within one hour of collection and stored at -20°C until batched analyses were performed. Echocardiography was repeated 7 days after beginning treatment (day 8). Venous blood collection, echocardiography, and biopsy of the semimembranosus muscle were also repeated 12 hours after completion of the treatment protocol (post-treatment, day 15).

#### **4.2.3 Treadmill Exercise**

Beginning on day 1 of the treatment period, horses were exercised on the Mustang 2000 high-speed treadmill<sup>f</sup> for a total of 15 minutes at a walk, trot, and fast canter. Treadmill exercise was performed once daily on weekdays (5 days per week) for 14 days. Skin sutures were removed from the biopsy site prior to beginning treadmill exercise. Speed for the walk ( $3.93 \pm 0.31$  mph) and trot ( $9.11 \pm 0.60$  mph) were determined subjectively based on each horse's stride length and perceived level of

exertion. Fast canter speed ( $19.66 \pm 1.27$  mph) was determined on the first day of exercise as the maximum speed at which the horse could keep pace with the treadmill for 2 minutes. Exercise intensity was gradually increased during the first week of the treatment period by increasing the time spent at a canter from 2 minutes on the first day to 5 minutes by day 8.

#### **4.2.4 Medication Administration**

Horses in the clenbuterol group (n=6) received clenbuterol as Ventipulmin® syrup ( $72.5\mu\text{g/ml}$ )<sup>g</sup> by mouth twice daily (8am and 8pm) for 14 days. The dose was incrementally increased during the treatment period to minimize adverse side effects, according to the following schedule:  $0.8\mu\text{g/kg}$  twice daily for 3 days, then  $1.6\mu\text{g/kg}$  twice daily for 3 days, then  $2.4\mu\text{g/kg}$  twice daily for 3 days, then  $3.2\mu\text{g/kg}$  twice daily for 5 days. Animals in the control group (n=6) received saline by mouth twice daily for 14 days. The volumes of saline administered each day were equal to the volumes of clenbuterol that would have been administered.

#### **4.2.5 Determination of Serum Clenbuterol Concentration**

Determination of serum clenbuterol concentrations was performed on thawed serum using gas chromatography following extraction with sodium hydroxide, petroleum ether and methylboronic acid. A standard curve using known concentrations of metoprololol from  $0.01 - 5.00\text{ng/ml}$  was generated in order to determine the limits of detection for this method. Thereafter, metoprololol was used as an internal standard to create a standard curve (linear regression) for each batch of samples tested, using known concentrations of  $0.25\text{ng/ml}$ ,  $0.50\text{ng/ml}$ ,  $1.00\text{ng/ml}$ ,  $2.50\text{ng/ml}$ ,  $5.00\text{ng/ml}$  and  $6.00\text{ng/ml}$ . The minimum acceptable  $R^2$  value for the resulting linear regression equation was set at 0.990.

#### **4.2.6 Evaluation of Skeletal Muscle Effects**

##### *4.2.6.1 Creatine Kinase and Aspartate Aminotransferase Activity*

CK and AST assays were performed on thawed (lithium) heparinized plasma by the LSU Clinical Pathology Laboratory using the Olympus AU36E analyzer<sup>h</sup>.

##### *4.2.6.2 Muscle Biopsy*

Horses were sedated intravenously with detomidine hydrochloride<sup>i</sup> and butorphanol tartrate<sup>j</sup> for biopsies. Local anesthesia with 2% lidocaine<sup>k</sup> was also instilled subcutaneously over the biopsy site. Pre-treatment biopsies were taken from the semimembranosus muscle approximately 5 cm below the

tuber ischii on the right side. Post-treatment biopsies were taken from the same muscle group on the contralateral side, 12 hours after the final treatment administration.

Briefly, a 1" (2.5cm) cube of muscle was surgically excised through a 2.5" (5cm) incision. The skin and subcutaneous tissues were closed in 2 layers with a simple continuous pattern using 2-0 nylon<sup>l</sup> and 3-0 polyglactin 910<sup>m</sup>, respectively. Horses were administered 2 grams of phenylbutazone<sup>n</sup> intravenously following each biopsy for analgesia and to minimize local inflammatory response.

A portion of the pre-treatment biopsy was wrapped immediately in a saline-moistened gauze sponge, placed in a water-tight container surrounded by ice packs, and analyzed histologically at an outside reference laboratory<sup>a</sup> to rule out pre-existing myopathy. The remaining portions of the pre-treatment biopsies, as well as all post-treatment biopsies, were fixed in zinc formalin and embedded in paraffin. For each horse, 2 paraffin blocks were prepared at each sampling point. From each available paraffin block, one 5µm-thick section was stained with hematoxylin and eosin, and 2 sequential 5µm-thick sections were cut for immunohistochemical staining.

#### 4.2.6.3 Histopathology

Muscle specimens stained with hematoxylin and eosin were examined at 400X magnification by a single blinded investigator (DBP). Slide-mounted specimens were evaluated for evidence of muscle damage using the scoring system outlined in Table 3.

**Table 3.** Scoring system for histologic evidence of muscle damage. Specimens were scored subjectively based on the degree of frequency and severity for each criterion.

CRITERIA	SCORE			
	0	1	2	3
Central nuclei	absent	rare	mild	moderate
Contraction band necrosis	absent	rare	mild	moderate
Intracytoplasmic granules	absent	rare	mild	moderate
Myocyte atrophy	absent	rare	mild	moderate
Myocyte fragmentation	absent	present		
Swollen myocytes	absent	rare	mild	moderate
Zenker's necrosis	absent	rare	mild	moderate



#### 4.2.6.4 Immunohistochemistry

The first of the 2 sequential muscle sections was evaluated for apoptosis using a polyclonal rabbit anti-caspase 3 antibody<sup>o</sup> with an avidin-biotin peroxidase complex system<sup>p</sup>. Briefly, sections were deparaffinized and rehydrated routinely with xylene and graded ethanol. Endogenous peroxidase activity was quenched by incubation in 100µl of 3% hydrogen peroxide for 10 minutes, followed by protein blockade with 100µl goat serum for 30 minutes. Sections were incubated in 100µl of a 1:1500 dilution of the primary anti-caspase-3 antibody for 30 minutes, followed by application of 100µl biotinylated anti-rabbit secondary antibody for 30 minutes, 100µl of avidin and biotinylated horseradish peroxidase complex for 30 minutes, and then 100µl of Nova Red peroxidase enzyme substrate<sup>q</sup> for 8 minutes. Specimens were rinsed in Tris buffer between each step. Lastly, counterstaining was performed with hematoxylin (100µl for 5 minutes), and specimens were cleared and dehydrated prior to permanent mounting.

The corresponding sequential muscle section from each block was used as a negative control by substituting deionized water for the primary anti-caspase 3 antibody in the first step of the procedure. Muscle sections from a case of clenbuterol toxicity reported in the previous chapter (horse 1) were also processed in the same manner for comparison.

Sections of equine laminae tissue from a patient with laminitis were used as positive controls<sup>67</sup>. Positive staining for caspase 3 was defined as bright red stain uptake in either the nucleus or cytoplasm of myocytes. Normal nuclei and sarcoplasm appeared various shades of purple. Muscle sections were examined for apoptosis by a single blinded investigator (JAT). The entire section was classified as either positive or negative for apoptosis based on presence or absence of positive (red) cytoplasmic or perinuclear staining. In addition, nuclear staining characteristics were recorded for 500 nuclei per specimen, and the percentage of positively-staining nuclei was recorded.

### **4.2.7 Evaluation of Cardiac Muscle Effects**

#### 4.2.7.1 Cardiac Troponin I Activity

Samples of K<sub>2</sub>EDTA plasma for cardiac troponin I (cTnI) analysis were shipped overnight on dry ice to the Clinical Pathology Laboratory at the University of Florida. Assays were performed on thawed EDTA plasma on the Bio-Site Triage MeterPlus, using Bio-Site Cardiac Panel cartridges<sup>f</sup>.

#### **4.2.7.2 Echocardiography**

Echocardiograms were performed using a 3.5MHz cardiac probe with a depth of 30cm attached to a portable ultrasound machine<sup>s</sup>. Hair was clipped over the right axillary region, and ultrasound coupling gel was applied to improve image quality. 2-D and M-mode analyses were performed in order to obtain standard measurements of cardiac function and contractility, specifically end-systolic and end-diastolic measurements of left ventricular internal diameter (LVIDs, LVIDd), interventricular septal thickness (IVSs, IVSd) and left ventricular posterior wall thickness (LVPWs, LVPWd), fractional shortening (FS), ejection fraction (EF), and diameters of the aortic root (AR) and pulmonary artery (PA). All echocardiograms were performed with horses at rest.

#### **4.2.8 Statistical Analysis**

The response variables CK, AST, cTnI, IVSd, IVSs, LVIDd, LVIDs, LVPWd, LVPWs, EF, FS, AR and PA were compared between treated horses and controls and across time periods using a Mann-Whitney U test and Friedman's test for repeated nonparametric data, respectively. An adjusted level of significance at  $p < 0.01$  was used to reduce type I error. These response variables were summarized as median and range.

The presence of apoptosis in muscle biopsy specimens was compared using a Cochran-Mantel-Haenszel stratified analysis, and the level of significance was set at  $p < 0.05$ . Percent apoptotic nuclei and serum clenbuterol concentrations were summarized as mean  $\pm$  standard deviation. All statistical analyses were performed using statistical software<sup>t</sup>.

### **4.3 Results**

#### **4.3.1 Treatment Groups**

Of 23 horses initially evaluated, 12 met the inclusion criteria. Of the 11 horses excluded, 1 was excluded due to reduced fractional shortening, and 10 horses were excluded based on abnormal muscle biopsy; 1 of these horses also had echocardiographic evidence of cardiac insufficiency. Horses in the control group (n=6) ranged in age from 3 to 10 years and included 2 mares and 4 geldings. The clenbuterol group (n=6) was comprised of 6 geldings between 4 to 7 years of age. There was no significant difference in age distribution between control and clenbuterol horses.

### **4.3.2 Effects on Skeletal Muscle**

#### *4.3.2.1 Creatine Kinase and Aspartate Aminotransferase Activities*

Prior to treatment (day 0), the median activity of CK in control group horses was 226 U/L (range, 174 – 286 U/L). The median activity of AST in control group horses was 242 U/L (range, 164 – 316 U/L). Among horses in the clenbuterol group on day 0, the median activities of CK and AST were 234 U/L (range, 112 – 384 U/L) and 243 U/L (range, 166 – 268 U/L), respectively.

On day 3, median activity of CK in the control group was 179 U/L (range, 141 – 215 U/L), and median activity of AST was 229 U/L (range, 167 – 289 U/L). In the clenbuterol group, median activity of CK was 175 U/L (range, 115 – 268 U/L), and median activity of AST was 214 U/L (range, 173 – 313 U/L).

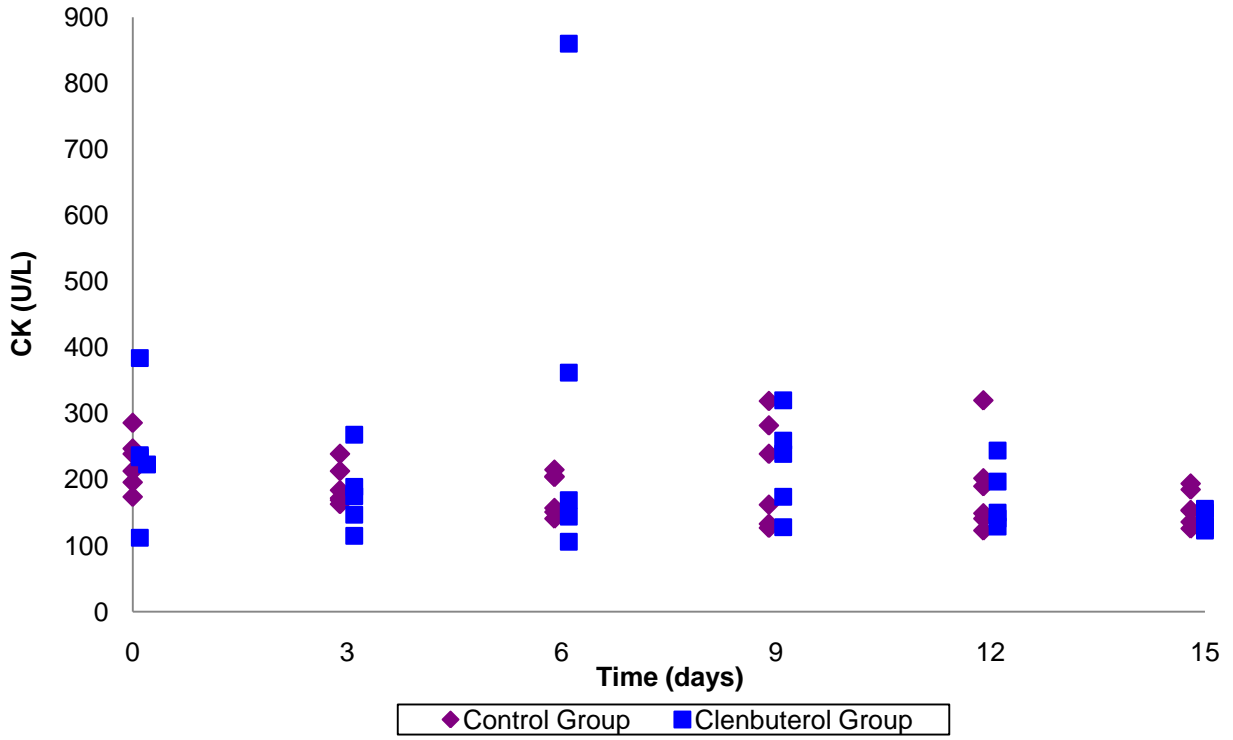
On day 6, CK activity above the reference interval was detected in 1 horse in the clenbuterol group, but this value did not significantly alter the overall CK activity of this group as compared to the control group. Median CK activity in the control group was 180 U/L (range, 141 – 215 U/L) versus 169 U/L (range, 106 – 860 U/L) in the clenbuterol group. Median AST activity in the control group was 228 U/L (range, 167 – 289 U/L), and median AST activity in the clenbuterol group was 202 U/L (range, 158 – 294 U/L).

Median activities of CK and AST on day 9 were 201 U/L (range, 127 – 319 U/L) and 238 U/L (range, 183 – 314 U/L), respectively, for horses in the control group; and 239 U/L (range, 128 – 320 U/L) and 198 U/L (range, 175 – 376 U/L), respectively, for horses in the clenbuterol group.

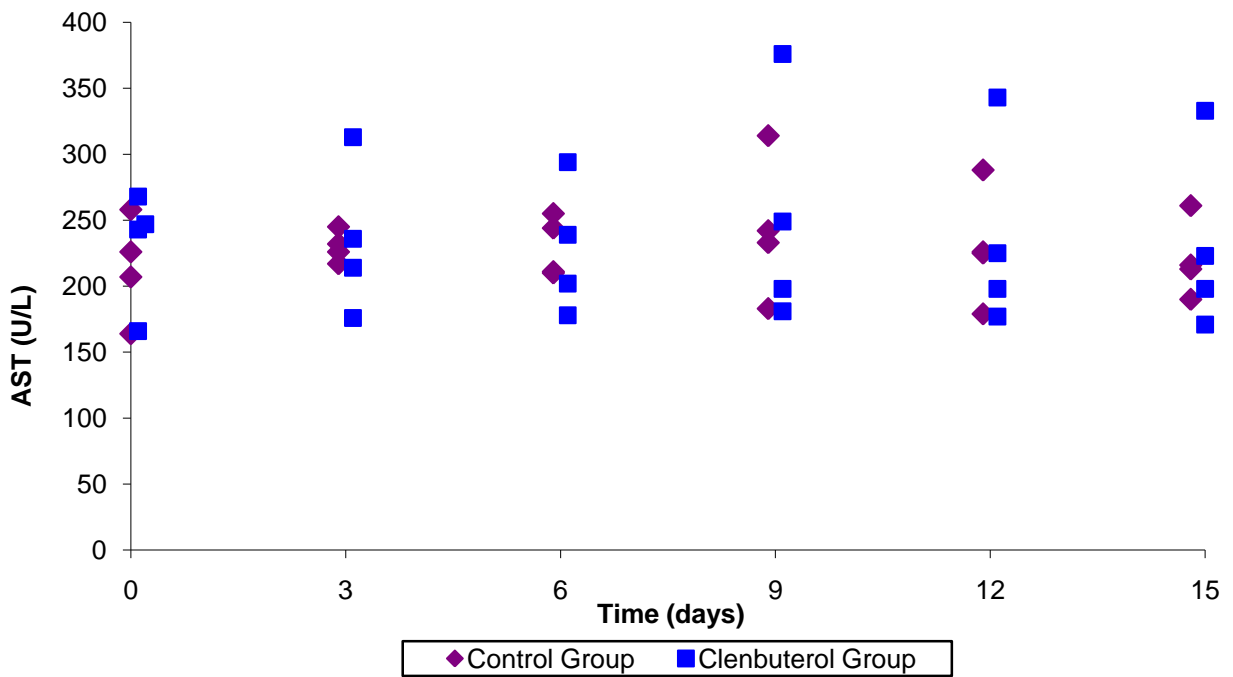
On day 12, median CK activity of the control group was 170 U/L (range, 123 – 320 U/L), and median AST activity of the control group was 226 U/L (range, 179 – 288 U/L). For the clenbuterol group, median CK activity was 150 U/L (range, 129 – 244 U/L), and median AST activity was 198 U/L (range, 176 – 343 U/L).

The medians of the post-treatment (day 15) activities of CK and AST were 154 U/L (range, 126 – 194 U/L) and 215 U/L (range, 172 – 261 U/L), respectively, for horses in the control group. For horses in the clenbuterol group, median CK activity was 129 U/L (range, 123 – 156 U/L), and median AST activity was 198 U/L (range, 171 – 333 U/L).

There was no significant difference in CK (Figure 4) or AST activities (Figure 5) between clenbuterol-treated horses and controls or across time periods for either treated horses or controls.



**Figure 4.** Scatter plot of CK activities (U/L). Values for clenbuterol-treated horses are denoted by the blue boxes (■), and values for saline-treated horses are identified by the purple diamonds (◆).



**Figure 5.** Scatter plot of AST activities (U/L). Values for clenbuterol-treated horses are denoted by the blue boxes (■), and values for saline-treated horses are identified by the purple diamonds (◆).

#### 4.3.2.2 Pre-Treatment Muscle Biopsy Classification

Of the 22 samples sent to the University of Minnesota for histologic evaluation, 9 were classified as normal muscle biopsies, 3 were suspicious for vitamin E deficiency, 4 were classified as having a non-specific myopathy, 5 were diagnosed with polysaccharide storage myopathy (PSSM), and 1 sample exhibited pathologic changes consistent with recurrent exertional rhabdomyolysis (RER).

#### 4.3.2.3 Histopathology

Ten horses had both pre- and post-treatment muscle biopsies available for sectioning. Post-treatment biopsies were unavailable for 1 horse from each group. Table 4 details the histologic scores for all biopsies examined. A trend towards a higher score for contraction band necrosis is apparent in the post-treatment biopsy specimens of horses treated with clenbuterol, as compared with control horses.

**Table 4.** Histologic scoring of muscle biopsy sections. For most morphologic criteria, scores range from 0 (absent) to 3 (moderate). Myocyte fragmentation is scored as 0 (absent) or 1 (present).

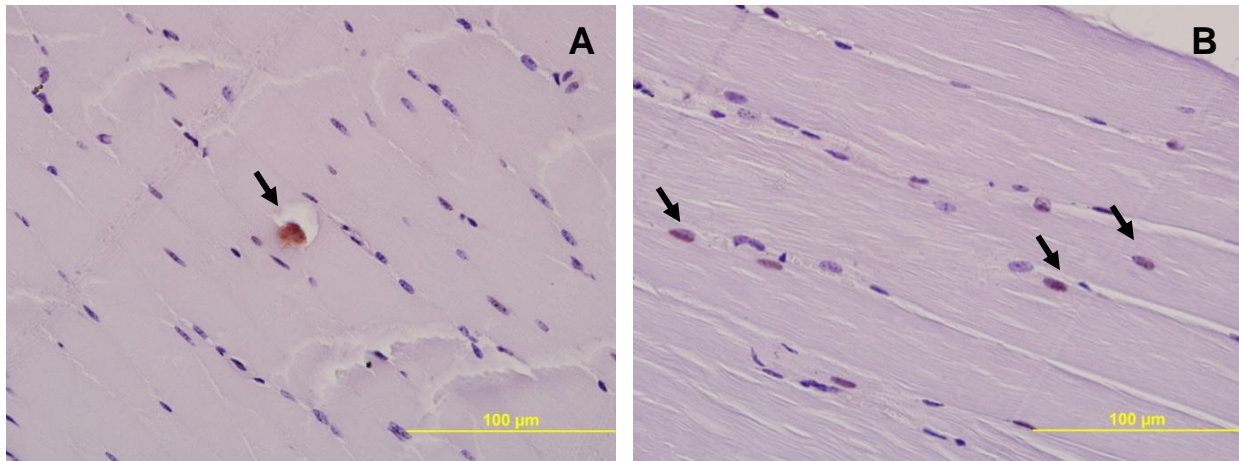
HORSE	1	2	4	5	7	8	9	11	18	21
GROUP	CON	CLB	CLB	CLB	CON	CON	CLB	CON	CLB	CON
<i>Central nuclei</i>										
PRE	0	1	0	0	0	0	1	1	1	0
POST	1	1	1	1	0	1	1	1	1	1
<i>Contraction bands</i>										
PRE	0	1	0	2	0	0	0	0	0	0
POST	0	1	3	2	0	0	2	0	3	0
<i>Intracyt. granules</i>										
PRE	1	2	2	1	0	0	2	1	2	3
POST	2	1	2	1	1	1	2	0	1	0
<i>Myocyte atrophy</i>										
PRE	1	2	1	1	0	0	2	1	2	1
POST	2	1	1	1	0	1	2	1	1	1
<i>Myocyte fragment.</i>										
PRE	0	0	0	0	0	0	0	0	1	0
POST	0	0	0	0	0	0	1	0	0	0
<i>Swollen myocytes</i>										
PRE	1	1	1	0	1	1	1	1	1	1
POST	1	0	1	0	1	1	1	0	1	2
<i>Zenker's necrosis</i>										
PRE	0	1	1	0	0	0	0	0	0	0
POST	1	0	1	1	0	0	1	1	0	0

\*CLB = clenbuterol group      \*CON = control group

\*PRE = pre-treatment (day 0)      \*POST = post-treatment (day 15)

#### 4.3.2.4 Immunohistochemistry

Ten horses had both pre- and post-treatment muscle biopsies available for sectioning. Post-treatment biopsies were unavailable for 1 horse from each group. No pre-treatment biopsies were classified as positive for cellular apoptosis. Three post-treatment sections from the control group and 2 post-treatment sections from the clenbuterol group displayed cytoplasmic staining (Figure 6A). Statistically, there was no significant interaction of group or time on the presence of apoptosis.



**Figure 6.** Semimembranosus muscle, 600X. Post-treatment caspase 3 immunohistochemical assays. The arrows denote positive Nova Red-staining apoptotic cells (A) and nuclei (B).

The mean percentage of caspase 3-positive nuclei in the pretreatment biopsies was  $24.6 \pm 7.56\%$  for the control group and  $19.0 \pm 7.91\%$  for the clenbuterol group. Post-treatment, the mean percentage of positively-staining nuclei for the control group was  $18.7 \pm 5.63\%$ , versus  $25.6 \pm 7.85\%$  for the clenbuterol group. Figure 6B depicts a specimen with a high percentage of positively stained nuclei. The observation of relatively frequent nuclear staining did not appear to change with exercise conditioning or clenbuterol treatment, unlike the observation of rare cytoplasmic staining. Considering that nuclear staining appeared to occur at a constant rate in all groups, and that caspase 3 is primarily a cytoplasmic enzyme, nuclear staining was interpreted as artifact.

### 4.3.3 Effects on Cardiac Muscle

#### 4.3.3.1 Cardiac Troponin I Activity

Activity of cTnI was elevated in 1 horse (5ng/ml) in the clenbuterol group on day 6. For this horse at all other time points, and for all other horses at all time points, cTnI activity was less than 0.05ng/ml.

There was no significant difference in cTnl between treated horses and controls or across time periods for either treated horses or controls.

#### 4.3.3.2 Echocardiography

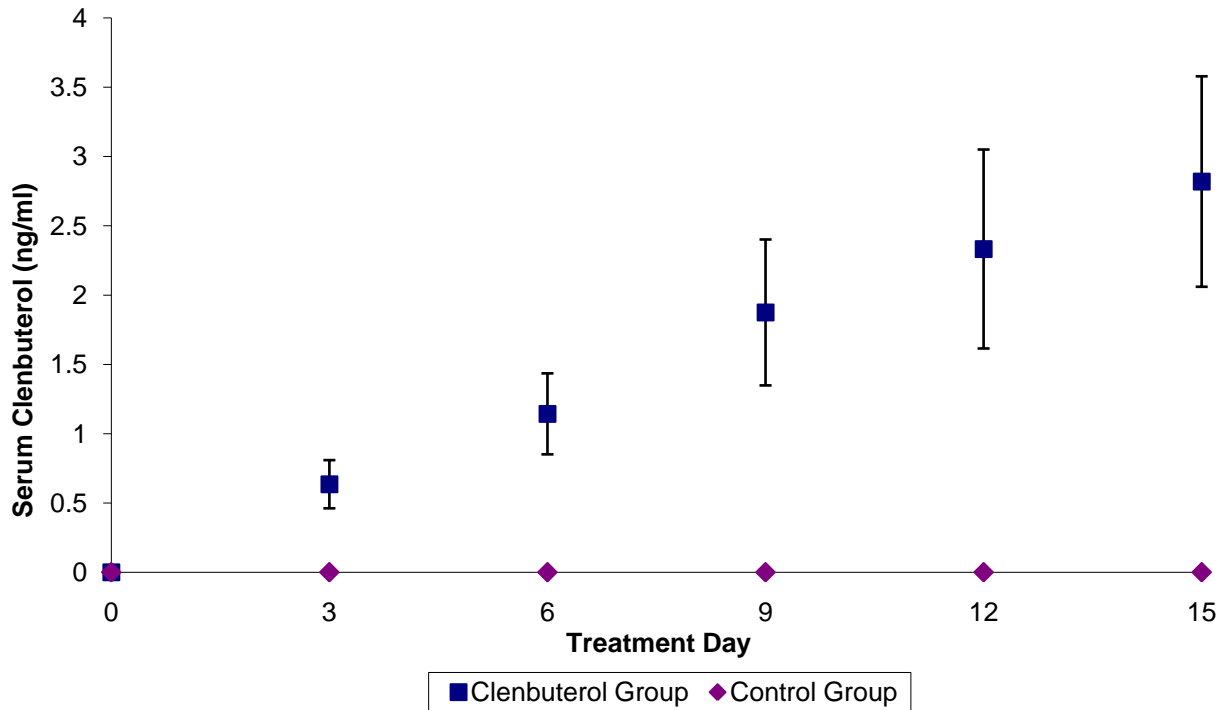
There was no significant difference between treated horses and controls or across time periods for either treated horses or controls in all variables examined (IVSd, IVSs, LVIDd, LVIDs, LVPWd, LVPWs, EF, FS, AR and PA). Table 5 summarizes the measured echocardiographic variables.

**Table 5.** Median values of measured echocardiographic variables. CLB = clenbuterol group.

	DAY 0		DAY 8		DAY 15	
	CONTROL	CLB	CONTROL	CLB	CONTROL	CLB
IVSd (cm)	3.18	2.99	3.20	3.02	2.99	2.89
<b>IVSs (cm)</b>	<b>4.58</b>	<b>4.57</b>	<b>4.56</b>	<b>4.55</b>	<b>4.49</b>	<b>4.42</b>
LVIDd (cm)	12.02	12.11	12.24	11.45	12.50	11.65
<b>LVIDs (cm)</b>	<b>7.75</b>	<b>7.21</b>	<b>7.76</b>	<b>7.35</b>	<b>7.25</b>	<b>6.69</b>
LVPWd (cm)	2.34	2.25	2.18	2.10	2.20	2.24
<b>LVPWs (cm)</b>	<b>3.48</b>	<b>3.94</b>	<b>3.55</b>	<b>3.71</b>	<b>3.74</b>	<b>3.66</b>
EF (%)	60.52	69.40	60.94	67.77	63.92	66.37
<b>FS (%)</b>	<b>35.49</b>	<b>41.71</b>	<b>36.48</b>	<b>40.20</b>	<b>38.30</b>	<b>42.10</b>
AR (cm)	7.36	6.97	7.24	7.37	7.50	7.45
<b>PA (cm)</b>	<b>4.64</b>	<b>4.36</b>	<b>4.86</b>	<b>4.71</b>	<b>4.74</b>	<b>4.58</b>

#### 4.3.4 Serum Clenbuterol Concentrations

The limit of detection for clenbuterol using gas chromatography was 0.25ng/ml (250pg/ml). Clenbuterol was not detectable in the serum of any horses prior to treatment (day = 0) or in the serum of saline-treated controls at any time point. For horses treated with clenbuterol, mean serum concentration of clenbuterol on day 3 was 635.2 ± 173.9 pg/ml. The morning of day 6, mean serum concentration was 1142.8 ± 292.1 pg/ml, and on day 9, mean serum clenbuterol levels were 1874.5 ± 526.4pg/ml. For days 12 and 15, mean serum clenbuterol concentrations were 2232.3 ± 717.7 pg/ml and 2819.0 ± 759.2 pg/ml, respectively. Figure 7 depicts the increase in serum clenbuterol levels during the treatment period.



**Figure 7.** Serum clenbuterol concentrations (ng/ml) increased in treated horses throughout the treatment period. Dots represent mean concentrations, and error bars represent 1 standard deviation.

#### 4.3.5 Adverse Events

Colic was the most frequently encountered adverse event, occurring in 3 horses. One horse in the clenbuterol group developed a large colon impaction and colon displacement on day 7 of treatment, necessitating early removal from the study. This horse was removed from final data analysis; therefore complete data were only available for 5 horses in the clenbuterol group.

One horse in the clenbuterol group developed a large colon impaction during the last week of the study, and one horse in the control group developed colic during the observation period after study completion. Both cases resolved within 24 hours with administration of flunixin meglumine and oral fluids. One horse in the control group developed diarrhea at the beginning of the study, which resolved within 48 hours after beginning treatment with di-trioctahedral smectite<sup>u</sup>, bismuth subsalicylate and metronidazole. One horse in the clenbuterol group experienced incisional dehiscence 7 days after pre-treatment biopsy, but the site healed well by second-intention after daily lavage, and the horse was able to begin treadmill exercise on schedule with no lameness.



Tachycardia, muscle tremors or other signs of clenbuterol toxicity were not recorded for any horse at any time point. Subjectively, horses in the clenbuterol group sweated more readily during treadmill exercise, and their perspiration did not decrease with conditioning, as it did in the control horses. Exercise tolerance was not specifically assessed, and all horses were able to complete the prescribed exercise protocol at all times.

#### **4.4 Discussion**

No adverse effects were associated with clenbuterol administration in the present study. The gradually increasing dose schedule used in this study, as recommended by the manufacturer, was intended to reduce the incidence of adverse effects; “tolerance” was likely induced due to progressive receptor down-regulation. Previous investigators reported that horses in their studies were unable to tolerate doses of clenbuterol higher than 2.4µg/kg every 12 hours; the reduction in sensitivity associated with receptor tachyphylaxis most likely accounts for the failure of this study to reproduce previously reported deleterious effects of clenbuterol.

##### **4.4.1 Effects on Skeletal Muscle**

The current study failed to find any association of clenbuterol or exercise conditioning with the selected indicators of skeletal muscle damage. The finding that CK did not increase with clenbuterol administration, particularly at higher doses, was unexpected and inconsistent with data reported by the manufacturer. However, publicly available information does not specify to what extent CK was reportedly elevated, nor at what time point following clenbuterol administration that CK elevation occurred. In clinical trials of human cardiac patients receiving clenbuterol at doses between 80 to 720µg/day, elevated CK activities were reported in approximately 50% of subjects<sup>3,34</sup>. It is possible that a change may have been detected with a different sampling time or a more frequent collection of samples. CK levels peak within 4 to 6 hours of muscle damage, but rapidly decline in the absence of further degeneration.

Blood samples were collected 15-20 hours post-exercise, when CK levels may have been returning to normal after potential exercise-induced injury. However, elevations in AST activity would still be expected under these circumstances. The sampling time in this study was chosen to correspond with the time that clenbuterol induces maximal myocyte necrosis in rats<sup>8</sup>. Fillies in race training are reported to have a higher prevalence of elevated CK and AST activity than colts<sup>73</sup>; the absence of females in the

clenbuterol group may have further reduced the probability of detecting alterations in these parameters. It is worth mentioning that in many of the studies which found negative effects of clenbuterol, the study population was comprised exclusively of mares<sup>5,6,74</sup>.

The semimembranosus muscle was chosen for evaluation because this muscle group is the optimal site to detect common equine myopathies on routine biopsy<sup>52</sup>. Unfortunately, this muscle is normally composed of 70-75% glycolytic (type IIX) fibers<sup>75</sup>, which are not the primary fiber types affected by clenbuterol in other species<sup>49</sup>, thereby limiting the probability of detecting an effect of clenbuterol. In murine studies, a significant difference in response to clenbuterol has been reported between skeletal muscles composed of predominantly fast-twitch fibers, those composed of mostly slow-twitch fibers, and cardiac muscle<sup>2,8,10,42,43</sup>.

Based on the findings of Burniston *et al*<sup>8-10,12</sup>, a high percentage of affected myocytes was not expected; however, even a small percentage (i.e., 2-3%) of apoptotic cells may result in significant tissue loss of up to 25% per day<sup>76</sup>. Most horses in this study lost weight during the treatment period. An initial loss of total body mass has been previously reported in horses subjected to clenbuterol administration plus exercise<sup>44</sup>; one explanation for this phenomenon is muscle tissue regression through apoptosis. Histologic evidence of myotoxicity appears to be attenuated after 8 days in other species<sup>8</sup>, so the 14-day treatment period used in this study may have further reduced the potential for detecting a significant histologic difference between groups.

Overall, the small sample size was detrimental to the detection of differences between groups. One barrier to obtaining a larger number of subjects was the unexpected high incidence of abnormal muscle biopsy results during the screening process. Approximately half of the animals examined were excluded from enrollment in the study based on abnormal muscle biopsies. Twelve out of 23 horses screened had one or more histopathologic abnormalities, and 10 of these were severe enough to require exclusion. Horses with pre-existing myopathy were excluded from this study, because elevations in serum CK and AST activities are commonly documented in horses with conditions such as PSSM and RER<sup>52</sup>, and inclusion of these horses in the study population could therefore confound interpretation of any such occurrences as being attributable to clenbuterol administration. Previous studies examining the effects of clenbuterol in horses<sup>5-7,44,45</sup> did not consider this factor.

Nevertheless, the high percentage (10/22, 45%) of horses affected with some form of histologically apparent myopathy was an unexpected finding. The stated reason for donation of most Thoroughbred horses to the research herd was poor racing performance; myopathy may be an under-recognized cause of poor performance in this population. In a retrospective analysis of causes of poor performance, 10/348 (3%) of horses were identified as suffering from rhabdomyolysis, and 53/348 (15%) of horses were diagnosed with subclinical myopathy based on abnormal post-exercise elevations in CK activity<sup>53</sup>. The horses in the current study were not evaluated for myopathy using this method, so direct comparison is difficult, but the prevalence of myopathy appears to be higher in our population than previously reported. Further investigation would be needed to clarify the relationship between these myopathies and poor performance, or response to clenbuterol in horses with and without myopathy. For the horses examined in the present study, history of clenbuterol administration during their racing career is unconfirmed but likely.

The effect of an inherent metabolic disorder of skeletal muscle on the potential for clenbuterol-induced myopathy is unknown, but one may speculate that its myotoxicity would be enhanced. For example, RER is thought to result from a calcium channel defect. Although one report in equine skeletal muscle did not find any change in calcium sensitivity in the fast-twitch muscle fibers of horses treated with clenbuterol<sup>48</sup>, other studies in rats concluded that myocardial calcium sensitivity<sup>36</sup> and sarcoplasmic calcium content<sup>33</sup> were increased with clenbuterol administration. Another study found that clenbuterol interferes with the ability of epinephrine to inhibit insulin-stimulated glucose uptake by skeletal muscle<sup>77</sup>. Whether this effect of clenbuterol increases the risk of rhabdomyolysis in horses already suffering from disorders of glucose metabolism (such as PSSM or metabolic syndrome) has not been investigated.

#### **4.4.2 Effects on Cardiac Muscle**

This study failed to find an association of exercise conditioning or clenbuterol administration with an increase in plasma cTnI or alterations in echocardiographic measurements at rest. Results of the study presented here agree with part of a previous study<sup>6</sup> that did not find an effect of clenbuterol on resting echocardiographic parameters or plasma activity of cTnI, when given for 8 weeks at 2.4µg/kg. However, Sleeper *et. al.* was able to demonstrate that significant changes in post-exercise echocardiography do occur with clenbuterol treatment, regardless of exercise conditioning<sup>6</sup>. Due to

limited data, this phenomenon was not observed in the present study. Only 1 horse in the clenbuterol group and 1 horse in the control group underwent weekly post-exercise echocardiographic evaluation during the treatment period, and a post-exercise echocardiogram was not performed in any horses during the enrollment period (day 0). The data available from these 2 horses is therefore insufficient for any significant statistical comparison.

One proposed mechanism for clenbuterol-associated cardiotoxicity is that of relative hypoxia, in which the myocardial oxygen demand is increased beyond achievable levels by the positive inotropic and chronotropic effects of clenbuterol<sup>7</sup>. Because treadmill speeds were arbitrarily assigned based on perceived exertion, rather than a quantitative measure of exercise intensity such as heart rate or  $VO_2$ , it is possible that the horses in our study did not exercise sufficiently to induce hypoxic necrosis. However, this explanation seems unlikely, as previous studies have shown exercise does not alter or potentiate other effects of clenbuterol<sup>6,44,45</sup>.

Cardiac troponin I is used in clinical practice as a non-invasive indicator of myocardial damage. Activity of cTnI increased outside of the reference range in only one horse at one time point, but changes were not statistically significant when considered along with the rest of the treatment group. Since echocardiography also failed to demonstrate significant changes in measured variables, we are unable to evaluate the sensitivity of cardiac troponin I for measuring cardiac injury in this population.

Other markers of cardiac injury in humans include B-type natriuretic peptide (BNP) and the cardiac isoenzyme of CK. Although total CK activities for horses in this study were within the reference interval, the relative activity of the cardiac isoenzyme versus the skeletal and brain isoenzymes was not specifically evaluated, and it may have been abnormal or different between groups. In human medicine, BNP is used as an indicator of left ventricular stretch in the evaluation of congestive heart failure, but it is less reliable as a monitoring tool than cardiac troponin activity, as levels may be increased by a myriad of non-cardiac causes such as renal disease, sepsis, acute respiratory distress syndrome or an increase in circulating catecholamines<sup>63</sup>. BNP appears promising in the dog for differentiation of congestive heart failure from non-cardiac causes of dyspnea<sup>78</sup>, but its clinical utility has not been validated in the horse.

Because the changes induced by clenbuterol appear to be clinically silent in the absence of exercise, future investigation into the effects of clenbuterol on cardiac function should incorporate a

standardized exercise test with documentation of heart rate and rhythm before, during and after exercise, as well as consistent post-exercise echocardiography. Monitoring of exercise tolerance may also be useful; methods were discussed in the previous section.

#### **4.4.3 Serum Clenbuterol Concentration**

Confirmation of serum clenbuterol concentrations in all treatment-group animals excludes the possibility that the lack of effects seen were due to inadequate drug administration. Previous reports on clenbuterol pharmacokinetics have not delineated the effects of gradually increasing doses on serum clenbuterol concentration. Two reports examining oral administration of 1.6µg/kg clenbuterol twice daily concluded that the 12 hour serum trough concentration in the adult horse was 508.1 ± 249.4 pg/ml<sup>25</sup> and 648.0 ± 189.7 pg/ml<sup>26</sup> after 2 and 7 days, respectively. The results obtained in our population on day 6 (1142.8 ± 292.1 pg/ml), following 2 days of oral clenbuterol administration at 1.6µg/kg, are higher than previously reported data. The reason for this discrepancy is unclear.

The dose of clenbuterol was held constant (at 3.2µg/kg PO q 12 h) from day 10 to day 14; therefore the increase in serum clenbuterol concentration from day 12 to day 15 was unexpected. It is possible that concurrent tissue clearance may have contributed to the continued elevation in serum concentration at the later sampling point.

#### **4.5 Product Information**

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<sup>a</sup> Neuromuscular Diagnostic Laboratory, University of Minnesota College of Veterinary Medicine, St. Paul, MN, USA

<sup>b</sup> Purina® Horse Chow® 200, Land O' Lakes Purina Mills, Gray Summit, MO, USA

<sup>c</sup> BD Vacutainer® Clot Tubes, Becton Dickinson, Franklin Lakes, NJ, USA

<sup>d</sup> BD Vacutainer® Heparin Tubes, Becton Dickinson, Franklin Lakes, NJ, USA

<sup>e</sup> BD Vacutainer® EDTA Tubes, Becton Dickinson, Franklin Lakes, NJ, USA

<sup>f</sup> Kagra Corporation, Fahrwangen, Switzerland

<sup>g</sup> Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA

<sup>h</sup> Olympus America Inc., Center Valley, PA, USA

<sup>i</sup> Dormosedan®, Pfizer Animal Health, Exton, PA, USA

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<sup>j</sup> Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA, USA

<sup>k</sup> Lidoject 2%, Butler Animal Health Supply, Dublin, OH, USA

<sup>l</sup> Ethilon™, Ethicon, Inc., Somerville, NJ, USA

<sup>m</sup> Vicryl™, Ethicon, Inc., Somerville, NJ, USA

<sup>n</sup> Butaject 20%, Butler Animal Health Supply, Dublin, OH, USA

<sup>o</sup> Affinity-Purified Rabbit Anti-Human/mouse Caspase 3 Active (AF835), R & D Systems, Minneapolis, MN, USA

<sup>p</sup> Vectastain® Elite® ABC Kit, Vector Laboratories, Burlingame, CA, USA

<sup>q</sup> Vector® NovaRED™ Substrate Kit, Vector Laboratories, Burlingame, CA, USA

<sup>r</sup> Bio-Site, Inc., San Diego, CA, USA

<sup>s</sup> GE Logiq Book Pro, Sound Technologies, Inc., Carlsbad, CA, USA

<sup>t</sup> SAS® Version 9.1.3, SAS Institute Inc., Cary, NC, USA.

<sup>u</sup> BioSponge™, Platinum Performance, Buellton, CA, USA

## CHAPTER 5. SUMMARY AND CONCLUSIONS

Oral clenbuterol administration at up to 3.2µg/kg twice daily did not produce measurable adverse effects on equine skeletal or cardiac muscle. However, as mentioned earlier, the small sample size limits the power of detection of any negative effects on skeletal or cardiac muscle. At supra-therapeutic doses, manifestations of toxicity in horses mimic signs reported in humans and can produce life-threatening complications. The β-adrenergic antagonist, propranolol, may be useful in symptomatic treatment of clenbuterol overdose in horses, but repeated administration may be required.

Although other studies in horses have demonstrated negative effects of low- to moderate-doses of clenbuterol on endurance and echocardiographic parameters, and produced measurable effects on lean muscle mass and fat mass, those studies identified changes after a much longer treatment period. Another study suggested that aerobic capacity was compromised 30 minutes after administration of a single dose of clenbuterol, but clenbuterol was administered intravenously in that study. The route and time course of administration of clenbuterol used in the current experimental study is different from all prior reports. It is possible that the significant changes detectable after 8 weeks of clenbuterol administration, or immediately after intravenous administration, may not occur after only 2 weeks of oral medication. Additionally, because aerobic capacity and exercise tolerance were not specifically assessed, this study neither confirms nor refutes previous data regarding the effects of clenbuterol on aerobic capacity or endurance.

Prolonged use of clenbuterol, particularly in the absence of therapeutic need, has been documented by previous researchers to have detrimental effects on many factors affecting athletic performance. Although these effects were not demonstrated in the present study, further investigation is needed to determine at what time point measurable changes occur in the skeletal and/or cardiac muscle, and if changes, once present, are reversible after discontinuing clenbuterol. This information would permit better recommendations to be made regarding the safety of prolonged and/or repeated clenbuterol administration. Because bronchiolar β-receptors are down-regulated after 1-2 weeks of clenbuterol administration, prolonged administration beyond this time results in decreased efficacy, unless the horse receives concurrent corticosteroid therapy or the clenbuterol dose is increased. Alternatively, clenbuterol may be given intermittently for symptomatic treatment, or repeated courses of treatment given after

respiratory  $\beta$  receptors have re-upregulated. As all of these practices are common in the performance horse industry, precise knowledge of the nature and extent of potential clenbuterol-induced injury is important to protect the welfare of the equine athlete. The cases of clenbuterol overdose reported herein underscore the concept that this medication is not completely benign.

However, at the dose, frequency, route of administration and duration of clenbuterol treatment used in the experimental study, negative effects on skeletal and cardiac muscle were not demonstrated. The dosing regimen used in this study is consistent with current recommendations for the use of clenbuterol in the treatment of IAD in young, athletic horses, assuming that symptoms resolve after a single course of treatment. Therefore, the results of this study suggest that clenbuterol, when administered orally for 2 weeks or less to horses with no pre-existing myopathy, does not induce skeletal or cardiac muscle damage. This finding is important, given the high prevalence and potential economic impact of IAD in performance horses. Further research is needed to assess the effects and potential for long-term skeletal and cardiac muscle damage associated with treatment periods longer than 14 days, and in horses with pre-existing myopathy.



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## VITA

Jessica Ann Thompson was born in January 1981 in San Antonio, Texas. She spent most of her childhood riding horses in the Dallas/Fort Worth area, but completed high school in Zionsville, Indiana in 1997. She returned to Texas for her undergraduate education and graduated *summa cum laude* from Texas Christian University in 2001 with a Bachelor of Science in biology and a Bachelor of Arts in chemistry. She began her veterinary education the same year at Texas A&M University and graduated *cum laude* from the College of Veterinary Medicine in 2005.

After receiving her Doctorate of Veterinary Medicine degree, Dr. Thompson married Miles Smith and moved to central Florida, where she completed a one-year internship in equine medicine and surgery at Equine Medical Center of Ocala, and got routinely sunburned in her spare time. In 2006, they relocated to Baton Rouge, where Dr. Thompson began a three-year combined residency in large animal internal medicine and Master of Science program at Louisiana State University School of Veterinary Medicine. She will complete her residency in July 2009 and will receive the Master of Science degree in veterinary medical sciences in August 2009.

Following graduation, she and her husband are looking forward to life's next big adventure, whatever that may be. They are not, however, looking forward to packing again.