LOCAL ADMINISTRATION OF BOTULINUM TOXIN TYPE-B IN THE EXTERNAL ANAL SPHINCTER OF HORSES PRODUCES TRANSIENT REDUCTION OF PEAK ANAL PRESSURE

By

David Adam-Castrillo, VMD

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

Master of Science
In
Veterinary Medicine

Nathaniel A. White II, Chair

Martin O. Furr
Lydia L. Donaldson
Kenneth E. Sullins

June, 2003
Leesburg, Virginia

Keywords: botulinum toxin, botulism, anal sphincter, equine, horse, perineal lacerations.

Copyright 2003, David Adam-Castrillo
LOCAL ADMINISTRATION OF BOTULINUM TOXIN TYPE-B IN THE EXTERNAL ANAL SPHINCTER OF HORSES PRODUCES TRANSIENT REDUCTION OF PEAK ANAL PRESSURE

David Adam-Castrillo, VMD

Abstract

Toxins produced by the Gram-positive bacteria Clostridium botulinum cause transient chemodenervation of mammalian muscle. The toxin binds to specific proteins within cholinergic presynaptic nerve terminals which regulate the release of acetylcholine in the synaptic space resulting in loss of muscle activation and function.

Local injections with botulinum toxins are currently used in humans for the treatment of disorders that benefit from prolonged neuromuscular blockade such as strabismus, blepharospasm, focal dystonias, spasticity, tremors, and anal fissures. Injections with botulinum toxin type A into the internal or external anal sphincter cause relaxation of the anal canal and allow healing of chronic anal fissures.

Perineal lacerations in mares, which occur during foaling often dehisce after surgical repair due to the high pressure across the incision resulting from accumulation of feces in the rectum. We hypothesized local injections of Clostridium botulinum type B toxin into the external anal sphincter could cause a decrease in anal pressures, thus reducing the incidence of dehiscence if used before surgical repair of perineal laceration in mares.

The purpose of this project was to determine the effects of BTB injection in the external anal sphincter in normal horses. Our hypothesis was that local injection of BTB would result in transient reduction of anal tone without causing clinical side effects. Peak
and resting anal sphincter pressures of horses were measured with a custom made rectal probe connected to a pressure transducer. Pressures were measured before treatment and after injection with Clostridium botulinum type B toxin (BTB) or saline. Dose titration with 500, 1000, 1500 and 2500 units of BTB was completed. The horses’ physical changes, behavior, and anal pressure were recorded. Injection of 1000 units of BTB produced significant reduction in peak anal pressure from days 2 to 84 when compared to control animals ($P<0.05$). Maximal effect of the toxin was observed within the first 15 days after injections followed by a slow return to baseline over 168 days. Injection in the anal sphincter with 2500 units of BTB in one horse produced signs of depression, generalized weakness, and dysphagia for 14 days. Clinical side effects were not observed in horses after injections with 500, 1000, or 1500 units of BTB.

In summary, local injections of botulinum toxin type-B in the external anal sphincter of horses caused transient relaxation of the anus and reduction of peak anal pressures. Systemic side effects were observed in one horse, which suggested a narrow dosage range to avoid toxicity. Further research to test the effects of botulinum toxin in clinical cases is needed to determine the full potential of this treatment modality.

This work was funded by Elan Pharmaceuticals.
DEDICATION

I would like to dedicate this research project to my father, for all the years of teaching and support and for his inspiring independent way of thinking.

Gracias Papá.
ACKNOWLEDGEMENTS

I would like to thank the following people for their contribution to this project:

-Dr. Nathaniel White II, my research and residency advisor, for his support of my ideas, for his patience, and for his help with the preparation of this thesis and research manuscripts.

-Dr. Lydia Donaldson, committee member, for her help with the design of the balloon probe, for supplying the monitoring equipment, and for her help with the preparation of the research manuscript.

-Dr. Martin Furr, committee member, for his input in the project design, and for his help with the preparation of the research manuscript.

-Dan Ward, for his assistance with the project design, and for the statistical analysis of the data.

-Dr. Marco Lopes, for his assistance writing the research proposal, and for his technical assistance and encouragement.

-Dr. Paula Guttman, for her technical assistance during the grueling process of data collection.

-Shawn Furr, our pharmacist, for her technical assistance.
# TABLE OF CONTENTS

**CHAPTER 1**  THE ANAL SPHINCTER-----------------------------------------------1  

1.1  INNERVATION OF STRIATED AND SMOOTH MUSCLE---------------------1  
1.2  ANATOMY AND INNERVATION OF THE ANAL SPHINCTER-------2  

**CHAPTER 2**  CLOSTRIDIAL BACTERIA-----------------------------------4  

2.1  THE GENUS CLOSTRIDIUM-------------------------------------------4  
2.2  BOTULISM IN HORSES-----------------------------------------------4  
2.3  ORIGIN, STRUCTURE, AND MECHANISM OF ACTION OF  
      BOTULINUM TOXIN---------------------------------------------------6  
2.4  REGENERATION OF THE NEUROMUSCULAR JUNCTION-----------------------8  
2.5  BACKGROUND HISTORY OF CLINICAL USE-----------------------------10  

**CHAPTER 3**  LOCAL ADMINISTRATION OF BOTULINUM TOXIN TYPE-B IN  
THE EXTERNAL ANAL SPHINCTER OF HORSES PRODUCES  
TRANSIENT REDUCTION OF PEAK ANAL PRESSURE----------------------12  

2.1  ABSTRACT----------------------------------------------------------12  
2.2  INTRODUCTION-------------------------------------------------------14  
2.3  MATERIALS AND METHODS-------------------------------------------15  
2.4  RESULTS-----------------------------------------------------------22  
2.5  DISCUSSION---------------------------------------------------------29  

**THESIS CONCLUSION**-----------------------------------------------------34  

**BIBLIOGRAPHY**-----------------------------------------------------------35-40  

**VITA**-----------------------------------------------------------------41
# TABLES AND FIGURES

## CHAPTER 3

<table>
<thead>
<tr>
<th>Figure 3-1</th>
<th>Picture of balloon probe</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3-2</td>
<td>Picture of pressure transducer</td>
<td>17</td>
</tr>
<tr>
<td>Figure 3-3</td>
<td>Picture of electronic monitor</td>
<td>17</td>
</tr>
<tr>
<td>Figures 3-4 and 3-5</td>
<td>Insertion of the probe in horses</td>
<td>18</td>
</tr>
<tr>
<td>Figures 3-6 to 3-9</td>
<td>Injection technique in the external anal sphincter</td>
<td>19</td>
</tr>
<tr>
<td>Figure 3-10</td>
<td>Peak and resting anal pressures after injection of 2500 U BTB versus saline (n=1)</td>
<td>23</td>
</tr>
<tr>
<td>Figure 3-11</td>
<td>Peak anal pressures after injection of 500, 1000, and 1500 U BTB versus saline (n=1)</td>
<td>25</td>
</tr>
<tr>
<td>Figure 3-12</td>
<td>Peak anal pressures after injection of 1000 U BTB versus saline (n=3)</td>
<td>27</td>
</tr>
<tr>
<td>Figure 3-13</td>
<td>Per cent change in baseline peak pressures after injection of 1000 U BTB versus saline (n=3)</td>
<td>28</td>
</tr>
</tbody>
</table>
CHAPTER 1
THE ANAL SPHINCTER

INNERVATION OF STRIATED AND SMOOTH MUSCLE

Somatic nerves originating in the ventral horn of the gray matter of the spinal cord innervate vertebrate skeletal muscle. Several muscle fibers innervated by a single motor neuron form a motor unit. At the nerve-muscle junction, there are specialized structures called synapses, where chemical transmission occurs. Action potentials travel from their site of origin along the axon towards the periphery spreading into all the terminal branches of the axon and motor end plates innervated by the motor unit. Pre-synaptic action potentials induce the activation of voltage-gated calcium channels in the membrane of the terminal, and calcium enters the nerve cell. A rise in calcium concentration initiates fusion of acetylcholine (ACh) containing vesicles with the presynaptic membrane, which results in exocytosis of Ach into the synaptic cleft. ACh binds postsynaptic specific receptors and ligand-gated ion channels open. There is a Na+ influx into the muscle cell which causes depolarization of the post synaptic muscle cell membrane. This depolarization opens voltage-gated Na+ channels around the synapse which generates an action potential that propagates to the entire muscle cell membrane. The subsequent release of stored calcium from the sarcoplasmic reticulum produces muscle contraction.\textsuperscript{1-2}

The autonomic nervous system is responsible for the extrinsic control of smooth muscle contraction, cardiac muscle contraction, and exocrine gland function. There are two branches of the autonomic nervous system: parasympathetic and sympathetic. In
contrast to the somatic nervous system, the autonomic nervous system has two peripheral nerves, a short pre-ganglionic nerve and a long post-ganglionic nerve. The neurotransmitter secreted in post-ganglionic parasympathetic nerves is ACh (also termed cholinergic synapses), whereas most post-ganglionic sympathetic nerves secrete norepinephrine (also called adrenergic synapses). ACh stimulates two types of receptors. 1) Muscarinic Ach receptors, which are stimulated by parasympathetic postganglionic neurons and cholinergic post-ganglionic sympathetic neurons. 2) Nicotinic receptors, which are found in all the autonomic synapses between pre and post-ganglionic nerves and at the somatic neuromuscular junction. Adrenergic receptors are subdivided into α and β in the basis of their effect when stimulated. The smooth muscle function of the gastrointestinal tract is intrinsically regulated by the enteric nervous system. There are stimulatory enteric neurons, which have ACh and substance P as regulatory neurotransmitters, and inhibitory enteric neurons, which contain neurotransmitters such as vasooactive intestinal peptide, somotostatin, nitric oxide and adenosine triphosphate.

3 ANATOMY AND INNERVATION OF THE ANAL SPHINCTER

The anus is the terminal part of the gastrointestinal tract. It is covered externally by thin, hairless integument provided with numerous sebaceous and sweat glands. Its lumen, the anal canal, is about 5 cm long in horses. The mucous membrane is glandless and covered with a thick squamous stratified epithelium. Musculature of the anus includes the internal anal sphincter, which is the terminal thickening of the circular smooth muscle coat of the bowel; and the external anal sphincter, which is a broad ring of
striated muscle fibers outside the internal sphincter.⁴⁻⁶ The internal anal sphincter receives parasympathetic innervation from the sacral spinal segments of the pelvic nerve and sympathetic innervation from spinal segments of the hypogastric nerve. Acetylcholine has a predominantly inhibitory effect on the internal anal sphincter smooth muscle via muscarinic receptors. Stimulation of β-receptors results in internal sphincter relaxation whereas stimulation of α-receptors results in inhibition. Contractions with noradrenaline and adrenaline can be converted to relaxations by the addition of α-receptor antagonists.⁷ Non-cholinergic, non-adrenergic neurotransmitters in the enteric nervous system such as nitric oxide, are responsible for intrinsic control of the internal anal sphincter. In addition, small populations of excitatory muscarinic receptors have been found in the internal anal sphincter.⁷ The external sphincter is solely innervated by general somatic efferent fibers in the pudendal nerve.³⁻⁵⁻⁷

Entry of feces into the rectum induces the “retrospincteric reflex, a reflex relaxation of the internal anal sphincter followed by peristaltic contractions of the rectum during the act of defecation. This reflex can be blocked by voluntary constriction of the external anal sphincter.⁶ The internal anal sphincter is responsible for 50-85% of the overall resting tone due to the combination of intrinsic myogenic activity and the extrinsic adrenergic innervation. The external anal sphincter has a greater contribution to the overall anal tone during periods of substantial rectal distension.⁷
CHAPTER 2
CLOSTRIDIAL BACTERIA

THE GENUS CLOSTRIDIUM

Clostridia are large, obligate anaerobic, spore-forming, rod-shaped bacteria that live in the soil and in the distal intestinal tract of most animals, including humans. The spores can be oval or spherical and often are greater in diameter than the rod itself. Vegetative forms of clostridia in tissue fluids occur singly, in pairs, or more rarely in chains. Infection may be acquired either by ingestion or wound contamination. Clostridial diseases can be divided into two groups: 1) gas-gangrene diseases, such as ‘black leg’ of cattle and sheep in which the organisms actively invade and reproduce in the tissues of the host with the production of toxins that enhance the spread of infection; and 2) diseases caused by toxemias resulting from the absorption of toxins produced by organisms proliferating in the intestines (enterotoxemias), in devitalized tissues (tetanus and botulism), or in food (botulism).

BOTULISM IN HORSES

There are seven distinct serotypes of botulinum neurotoxins (BoNT) (A-G) produced by strains of Clostridium botulinum, C. butyricum, or C. barati. Horses are most commonly affected by type B, and occasionally by type C or type A. “Forage poisoning” is the most common form of botulism in adult horses. The onset of the disease may be acute, with rapid progression of diffuse paralysis and dyspnea, leading to recumbency and death. Slower onset and progression may also occur over several days.
Clinical signs are the result of impaired cholinergic neurotransmission. Common signs of intoxication include generalized weakness, trembling, inability to fully retract the tongue, drooling of saliva, flaccidity of the tail, dysphagia, and sluggish papillary light reflexes. Rapid onset of clinical signs often results in death, whereas gradual onset can be associated with complete recovery over a period of weeks to months. The rate of progression of clinical signs after ingestion of contaminated feed depends on the dose of toxin. Less commonly, absorption of botulinum toxin occurs via contaminated wounds or secondary to an injection abscess. Foals ranging from 2 weeks to 8 months of age are typically affected by “toxicoinfectious botulism,” where botulinum toxins are produced by bacteria growing in the intestinal tract. Intoxication produces the “shaker foal syndrome,” characterized by paresis, stiff and stilted gait, and generalized muscle trembling. Rapid progression of the disease leads to respiratory paralysis and death.\textsuperscript{12}

Treatment for botulism is mainly symptomatic, and involves fluid and nutritional support, nursing care, and ventilatory support if necessary. Polyvalent antitoxin is useful in the early stages of the disease to neutralize any unbound toxin. Antibiotic therapy is indicated in cases of wound and toxicoinfectious botulism. Antibiotics of choice are metronidazole and potassium penicillin. Antibiotics that potentiate neuromuscular weakness such as aminoglycosides, tetracyclines, and procaine penicillin should be avoided. Control measures to prevent the disease include the avoidance of contaminated food or water and immunoprophylaxis. An effective toxoid vaccine is available for \textit{C. botulinum} type B in horses.\textsuperscript{12}
ORIGIN, STRUCTURE, AND MECHANISM OF ACTION OF BOTULINUM TOXIN

Toxins are produced when bacteria contain specific neurotoxin genes. These genes are mobile and non-toxic bacterial strains can become toxigenic by gene transfer facilitated by phages, plasmids, or conjugation transposomes. Bacterial strains can produce more than one BoNT type if they contain more than one toxigenic gene.\(^{10-11,13}\) BoNT are translated as single, inactive, polypeptide of 150 kDa in the bacterial cytosol.\(^{10-11,13-17}\) Only after bacterial lysis, BoNT are released in the form of very stable, high molecular mass complexes of up to 900 kDa composed of toxic and non-toxic proteins called progenitor toxins. Aggregation of BoNT is important to prevent proteolysis and denaturation during exposure to low temperature, solvent removal, or low pH.\(^{10,17}\) When the progenitor toxins reach the small intestine, the slightly alkaline pH induces their dissociation and individual BoNT reach the general circulation by transcytosis from the apical to the basolateral side of the intestinal epithelial cells or by uptake from M cells. Rarely, BoNT reach the general circulation through wound contamination.\(^{10}\) Tissue proteases cleave the inactive polypeptide within a surface exposed loop subtended by a disulphide bridge resulting in an active di-chain neurotoxin composed of a 50 kDa light chain (L chain) and a 100 kDa heavy chain (H chain) linked by non-covalent protein-protein interactions and a conserved S-S bond.\(^{10-11,13-17}\) Differences in length of the polypeptide chains have been observed among the various BoNT serotypes, which present homologous segments separated by regions of little or no similarity.\(^{10}\)

Activated BoNT diffuse in the body fluids and bind to presynaptic terminals of cholinergic neurons where they become internalized into the nerve cell cytosol inside
acidic vesicles. Translocation of BoNT from the acidic vesicles to the neuronal cytosol is a process that is not fully understood. The “cleft” model, which explains all available experimental data, proposes that at low pH the H and L chains change their conformation in such a way that both of them expose hydrophobic surfaces and enter into contact with the hydrophobic core of the lipid bilayer. The H chain forms a transmembrane hydrophilic cleft that allows the passage of the partially unfolded L chain. Exposure to the neutral pH of the cytosol induces the L chain to refold. Reduction of the interchain disulfide bond results in the release of the L chain in the cytoplasm. The L chain contains a zinc-binding zinc-endopeptidase motif that targets specific proteases called SNARE (Soluble N-ethylmaleimide sensitive factor Attachment protein REEeceptor) proteins. These proteins are essential components of the vesicle exocytosis machinery, which is a highly conserved process within mammalian species. There are three synaptic SNARE proteins all of which share a unique nine-residue motif, called the SNARE motif. The three small peptides are syntaxin, SNAP-25, and synaptobrevin or VAMP (Vesicle-Associated Membrane Protein). Syntaxin and SNAP-25 are plasma membrane-associated proteins. There are two copies of the SNARE motif in VAMP and syntaxin, and four copies in SNAP-25. BoNT serotypes have selective affinity for SNARE proteins: types A, C, and E target SNAP-25; types B, D, F, and G target VAMP; and type C targets syntaxin and SNAP-25.

In addition to the SNARE proteins there are other proteins involved in the machinery of exocytosis. These include the ATPase NSF (N-ethylmaleimide Sensitive Factor) and its adaptor SNAP (Soluble NSF Attachment Protein), the Rab class of small G proteins and their effectors, the synaptotagmin family, the nSec1 family, and other
factors such as complexin, VAP33, and synaptophysin. The initial steps of exocytosis involve the binding of syntaxin to nSec1. The syntaxin/nSec1 complex is dissociated by a Rab effector at the docking stage and synaptobrevin in the docked vesicle then binds to syntaxin and SNAP-25 in the cytosolic side of the plasmalemma. Fusion of the vesicles with the plasma membrane occurs with a local increase of $Ca^{2+}$ resulting after depolarization of pre-synaptic voltage-gated channels during the transmission of a nerve impulse. At the recycling stage, a-SNAP and NSF bind to the SNARE complex, and the SNARE complex is dissociated in an ATP-dependent hydrolysis. Cleavage of SNARE proteins by BoNT prevents the formation of SNARE complexes, thus halting the docking and fusion of ACh containing vesicles with the plasmalemma. With electron microscopy of motor end plates after BoNT intoxication, accumulation of many small synaptic vesicles on the cytosolic side of the plasma membrane is demonstrated. Blockade of neuromuscular transmission and muscle paralysis occurs due to lack of release of ACh into the synaptic space.

**REGENERATION OF THE NEUROMUSCULAR JUNCTION**

No apparent loss of motor axons is observed subsequent to BoNT intoxication. Muscle fibers undergo progressive atrophy with reduction in mean diameter 2-6 weeks after BoNT exposure due to lack of nerve stimulation of the muscle. There is a BoNT-induced synapse remodeling mediated by large increases of the growth factor calcitonin gene-related peptide (CGRP), which is accumulated in vesicles at the terminal. In addition, denervated muscle fibers release insulin-like growth factors IGF-I and IGF-II which upregulate CGRP synthesis. These growth factors induce enlargement of
the motor end plate and the formation of sprouts that grow from the end plate and the
terminal part of the axon at the nodes of Ranvier in the direction of muscle fibers. The
number of motor end plates on a single muscle fiber also increases. Nerve sprouting and
formation of functional nerve-muscle junctions allow for gradual return of normal muscle
function. During a second phase of the rehabilitation process, synaptic activity to the
original nerve terminals returns. Because nerve sprouts are not as efficient in mediating
exocytosis and endocytosis as the parent nerve terminals, the muscle responds by
signaling sprout elimination or by turning-off sprouting signals and the sprouts eventually
disappear.\(^\text{23}\) The motor end-plate regeneration process takes 2-4 months in mammalian
species.\(^\text{14}\)

Recovery from BoNT poisoning depends on the ability of the motor end plate to
produce new functional SNARE proteins after BoNT are degraded. BoNT types A and E
both cleave SNAP-25 but at different sites.\(^\text{24-25}\) The effect of BoNT/E is reversed much
sooner (30 days) than BoNT/A (90 days). This is because the truncated, non-functional
BoNT/E SNAP-25 molecules are rapidly removed from the cytosol and rapidly replaced
by newly synthesized SNAP-25. Truncated BoNT/A SNAP-25 molecules are non-
functional for exocytosis but preserve structural functions and are removed and replaced
slowly.\(^\text{10,22-23}\) VAMP-targeting BoNT (B, D, F, and G) give rise to relatively short
periods of paralysis.\(^\text{17,19,23-24,26-27}\)

**BACKGROUND HISTORY OF CLINICAL USE**

Botulinum toxin type A was first used as an injectable selective muscle
weakening agent in humans in the late 1970s and early 1980s after it was investigated in
monkeys in the late 1960s and 1970s. The goal was to provide a pharmacological alternative to surgery in non-accommodative strabismus by injection of botulinum toxin in the extraocular muscles. The toxin produced alteration of ocular alignment lasting 2-8 months. From these early studies it was established that botulinum type A injections were an effective non-surgical alternative treatment for strabismus. In 1989 type A toxin (Botox; Allergan Pharmaceuticals, Irvine, CA) was approved by the FDA for the treatment of strabismus, blepharospasm, and hemifacial spasm. Botox was later also approved for cervical dystonia and glabellar wrinkles. Type B toxin (Myoblock: Elan Pharmaceuticals, San Diego, CA) was approved for cervical dystonia in 2000 and more recently for glabellar wrinkles.

Botulinum toxin therapy is used in humans for a wide variety of spastic and painful syndromes such as cerebral palsy, cervical dystonia, back pain, chronic headaches, chronic anal fissures, focal hyperhydrosis, esophageal achalasia, blepharospasm, adductor spasmodic dysphonia, and for the diagnosis of sphincter of Oddi dysfunction. The toxin is effective by alleviating hypertonicity of muscles and muscle sphincters, muscle spasms, and extensor and flexor reflexes. New evidence suggest than botulinum toxin may be involved in mediation of neuropathic pain. With chronic neuropathic pain stimuli that are normally not painful produce pain. This phenomenon is thought to be due a reorganization of the nervous system after injury. Botulinum toxin may reduce neuropathic pain by altering the peripheral mechanism of neuropathic pain transmission through a direct effect on non-cholinergic neurons, resulting in reduced release of substances such as glutamate and substance P.
The main problems observed with botulinum toxin therapy are the secondary formation of antibodies and local diffusion of toxin to neighboring muscles. Accurate frequency of secondary non-responsiveness is not known but high amounts of toxin used per treatment and frequent treatments contribute to a greater risk for antitoxin antibody formation. However, because any portion of the botulinum protein preparation is a potential antigen, antibodies can form against non-toxin proteins. Botulinum toxin preparations contain about 20% of the actual neurotoxin, which explains why only a small subpopulation of antibodies derived from an injected preparation is capable of neutralizing the toxin.

Local injection of botulinum toxin has not been reported in the horse. The sensitivity of horses to botulism suggests it would cause local paralysis but that systemic effects may be observed. This study was completed to test possible clinical application using the anal sphincter as a muscle of clinical importance during repair of the perineal lacerations in mares.
CHAPTER 3
LOCAL ADMINISTRATION OF BOTULINUM TOXIN TYPE-B IN THE EXTERNAL ANAL SPHINCTER OF HORSES PRODUCES TRANSIENT REDUCTION OF PEAK ANAL PRESSURE

ABSTRACT

Objective- To study the effects on anal sphincter tone following local injection with Clostridium botulinum toxin type B.

Animals- 11 healthy adult horses.

Procedure - Peak and resting anal sphincter pressures were measured with a custom made rectal probe connected to a pressure transducer. Pressures were measured before treatment and after injection with Clostridium botulinum type B toxin (BTB) or saline. Dose titration with 500, 1000, 1500 and 2500 units of BTB was completed. The horses’ physical changes, behavior, and anal pressure were recorded.

Results- Injection of 1000 units of BTB produced significant reduction in peak anal pressure from days 2 to 84 when compared to control animals (P<0.05). Maximal effect of the toxin was observed within the first 15 days after injections followed by a slow return to baseline over 168 days. Injection in the anal sphincter with 2500 units of BTB in one horse produced signs of depression, generalized weakness, and dysphagia for 14 days. Clinical side effects were not observed in horses after injections with 500, 1000, or 1500 units of BTB.

Conclusions and Clinical Relevance- The effect of focal intramuscular injection of BTB in horses is similar to other species. However horses, compared to other species, appear
to be more sensitive to BTB and clinical signs of botulism may develop at doses exceeding 1500U. Injections with BTB in the external anal sphincter of mares may be useful to reduce incisional dehiscence after repair of perineal lacerations.
INTRODUCTION

Toxins produced by the Gram-positive bacteria *Clostridium botulinum* cause transient chemodenervation of mammalian striated muscle. The toxin binds to specific proteins within cholinergic presynaptic nerve terminals, which regulate the release of acetylcholine in the synaptic space resulting in loss of muscle activation and function. Duration of effect is dependent on regeneration of new motor end plates, which takes 2-4 months in mammalian species. Local injections with botulinum toxins are currently used in humans for the treatment of disorders that benefit from prolonged neuromuscular blockade such as strabismus, blepharospasm, focal dystonias, spasticity, tremors, and anal fissures.

Anal fissures in humans heal poorly due to increased tension of the fissure edges and decreased blood flow. One or two injections with botulinum toxin type A (BTA) into the internal or external anal sphincter cause relaxation of the anal canal and allow healing of chronic anal fissures. Perineal lacerations in mares which occur during foaling often dehisce after surgical repair due to the high pressure across the incision resulting from accumulation of feces in the rectum. We hypothesized that local injections of *Clostridium botulinum* type B toxin (BTB) into the external anal sphincter could cause a decrease in anal pressures, thus reducing the incidence of dehiscence if used before surgical repair of perineal laceration in mares.

The purpose of this project was to determine the effects of BTB injection in the external anal sphincter in normal horses. Our hypothesis was that local injection of BTB would result in transient reduction of anal tone without causing clinical side effects.
Materials and Methods

Eleven adult healthy horses were used in the study. Horses included 1 Thoroughbred (TB) mare, and 10 geldings (2 TB, 3 Quarter Horse, 2 Warm Blood, 1 Arabian, and 2 Paint). Mean weight and age was 586.2 kg (range, 488.6 to 676.4 kg) and 12.2 years (range, 7 to 24 years), respectively. All horses were kept on pasture for the duration of the study. The horses did not have a history of vaccination with Clostridium botulinum toxoid for at least a year before the experiment. Horses were restrained in stocks during injection and measurement of anal tone. Water and sweet feed was offered to the horses during measurements to evaluate their ability to swallow.

A probe designed to determine anal sphincter tone was made from an endotracheal tube\(^a\) modified by inserting two rubber rings at the tip of the endotracheal tube in order to keep the cuff within the anal canal during measurements (Figure 3-1). The equine anal canal has an average length of 5cm\(^4\) and therefore the length of the cuff and the distance between the rings was set to 5cm. The endotracheal tube was connected to an electronic pressure transducer\(^b\) and monitor\(^c\) to measure anal sphincter pressures (Figures 3-2 and 3-3). The electronic pressure transducer was calibrated to a mercury manometer before and after the experiments. For each horse the pressure transducer was set to zero when the cuff was filled with 10 ml of water before each of the measurements. Horses were restrained in stocks and peak and resting anal sphincter pressures were measured (Figures 3-4 and 3-5). Peak pressures were recorded when reflex constriction of the

---

\(^a\)Aire Cuff®, 24mmOD, Bivona Inc., Gary, Indiana.
\(^b\)Gould P23 ID, Oxnard, California.
anal sphincter was induced with slight rotation of the rectal probe. Baseline peak and resting pressures were recorded before the treatments were administered. Five measurements of peak and rest anal pressures were taken from each horse when they were standing quietly in the stocks.

Figure 3-1. Photograph of the probe used to measure anal pressure in horses.
Figure 3-2. Photograph of the pressure transducer, which connected to the rectal probe.

Figure 3-3. Photograph of the electronic monitor used to obtain anal pressure measurements.
The average from the five measurements was used for data analysis. Once the baseline pressures were obtained the horses were sedated with xylazine hydrochloride and restrained with a nose twitch. The perianal region was thoroughly scrubbed with chlorhexidine soap solution. Treatments were randomly assigned. Syringes which contained either 4 ml of sterile saline for control animals or one of three different doses of botulinum toxin type B (BTB) with a total volume of 4ml were prepared for each of the experiments. The investigators were blinded to all treatments. Injection of the toxin was completed by inserting two fingers into the anus to stretch the dorsal part of the anal sphincter caudad (Figures 3-6 to 3-9).

Sedazine, Fort Dodge Animal Health, Fort Dodge, Iowa.
Nolvasan Solution, Fort Dodge Animal Health, Fort Dodge, Iowa.
Myoblock®, Elan Pharmaceuticals, San Francisco, California.
A 22 gauge x 1.5 inch needle was inserted in the external anal sphincter muscle to the level of the hub. The contents of the syringes were slowly injected as the needle was gradually removed from the muscle. Three separate experiments were performed to evaluate the effect of intramuscular injections of BTB in the external anal sphincter.

**Figures 3-6 to 3-9.** The injection technique is demonstrated. The needles are inserted at the 10 and 2 o’clock positions into the external anal sphincter. Four milliliters of either saline or botulinum toxin type B solution are injected at each site.

**Experiment I**- For the initial evaluation a single injection of 2500 units of BTB was injected at the 12 o’clock position in the external anal sphincter in one horse.
(4.4U/kg). A control horse received an equal volume of saline injected in the same position. Anal pressures were measured at days 1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 14, 17, 21, 28, 42, 56, 70, 84, 98, 112, 126, 140, 156, and 168 in the horse treated with the toxin, and on days 1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 14, 17, 21, 28, and 42 in the control horse. Swallowing and anal, tail, upper eyelid, and tongue tone were monitored daily for the first 28 days and thereafter at the time of rectal pressure measurements. Temperature, heart rate, and respiratory rate were measured at the time of anal pressure measurements up to day 21.

**Experiment II**- Three horses received 500 (.88U/kg), 1000 (1.75U/kg), and 1500 (2.20U/kg) units of BTB respectively. One horse used as a control received sterile saline. Two injections were made at the 10 and 2 o’clock positions in the external anal sphincter of each of the horses. Anal pressures were monitored at days 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 42, 56, 70, 84, 98, 112, 126, 140, and 168. The horses were monitored for swallowing, and for anal sphincter, tail, upper eyelid, and tongue tone. Temperature, heart rate, and respiratory rate were measured for the first three days after the injections.

**Experiment III**- The anal sphincter tone was evaluated in six horses. Three control horses, including the control horse from experiment I, received sterile saline and three horses received a total dose of 1000 units of BTB (1.70-2.05U/kg) in the anal sphincter. The injection protocol was the same as for experiment II. Anal pressures were monitored at days 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 42, 56, 70, 84, 98, 126, 140, 154, and 168. Horses were monitored as in experiment II.
**Statistical analysis** - Because baseline peak pressures were different among horses, results were compared on a per cent from baseline. A statistical program\(^8\) was used to test the influence of treatment on each day after treatment using repeated measures of ANOVA. The Bonferroni correction was applied. A value of \(P<0.05\) was considered significant.

Results

Experiment I-. The horse injected with 2500 units of BTB had a 49.4% reduction of peak anal pressure on day 2 and a maximal reduction of 89% on day 11 compared to baseline (Figure 3-10). Return to 94% of baseline peak pressure was recorded on day 154. There was no change in the peak pressures in the control horse. On day 10 the horse that received BTB demonstrated mild generalized weakness, low head carriage, diarrhea, and dysphagia (water reflux from the nostrils when drinking, prolonged chewing, hyper salivation, and partial dropping of grain). On day 24 signs of dysphagia and all other neuromuscular deficits resolved. On day 4, the dorsal hemi-circumference of the anus was very thin with digital palpation compared to the ventral portion of the sphincter in the horse treated with BTB. Return to normal sphincter thickness occurred gradually over 98 days after BTB injection. There were no complications at the injection sites in either horse.
Figure 3.10. Resting and peak anal pressures of two horses after local injections in the external anal sphincter with saline (placebo) or 2500 units of BTB.
Experiment II. Horses treated with BTB had a mean reduction of 37.8 and 56.3% from baseline peak anal pressures compared to baseline on days 2 and 5, respectively (Figure 2-11). The greatest mean drop in peak anal pressure (63.6%) was observed on day 15. An average return to 98.6% of baseline peak anal pressures was observed by day 168. Resting pressures did not change after toxin or placebo treatments. The horse injected with 1000 units BTB (1.75 units/kg) had the greatest drop in peak anal pressures compared to baseline (46.4, 74.8, and 81.7%, on days 2, 5, and 15, respectively) (Figure 3-11). On days 2 and 4, ¾ of the dorsal circumference of the anus was very thin compared to the ventral portion of the sphincter in horses receiving 1000 and 500U of BTB, respectively. Return to normal sphincter thickness occurred gradually 56 to 84 days after treatment. The horse receiving 1500U of BTB did not develop a distinct area of decreased thickness around the injection sites. None of the horses injected with toxin developed clinical side effects or local reactions at the site of injections.
Figure 3-11. Peak anal pressures in each of four horses after local injections in the external anal sphincter with saline (placebo), or 500, 1000, and 1500 units of BTB.
**Experiment III** - Horses appeared to have a variable response to the same dose of BTB (Figure 3-12). Horses injected with BTB had a mean reduction of 46.7 and 60.3% from baseline peak anal pressures compared to baseline on days 2 and 5, respectively (Figure 3-13). The greatest drop in peak anal pressure (68%) was observed on day 15 (Figure 3-13). An average return to 96.2% of baseline peak anal pressures was observed on day 168 (Figures 3-12 and 3-13). There was a significant difference ($P<0.05$) in peak anal pressures between toxin treated horses and controls from days 2 to 84 (Figure 3-13). Peak pressures of BTB treated horses were lower than that of control horses from days 84 to 140, but results were not statistically significant. Resting pressures did not change after toxin or placebo treatments. Between days 2 and 3 after injections, ¾ of the dorsal circumference of the anus was very thin compared to the ventral portion of the sphincter in all horses receiving BTB. Return to normal sphincter thickness occurred gradually 56 to 84 days after treatment. None of the horses injected with toxin developed clinical side effects or local reactions at the site of injections.
Figure 3-12. Peak anal pressures of six horses after local injections in the external anal sphincter with saline (placebo) or 1000 units of BTB.
Figure 3-13. Per cent change from baseline peak anal pressures after local injections in the external anal sphincter with saline (placebo, n=3) or 1000 units of BTB (n=3). Asterisks indicate significant differences ($P<0.05$) between toxin treated horses and controls.
Seven types of Clostridium botulinum neurotoxins have been identified: A, B, C, D, E, F, and G. The mechanism of action of botulinum toxin occurs in four steps: 1) toxin binding to presynaptic membranes of peripheral nerve terminals. 2) internalization of toxin into endocytic vesicles leading to the formation of an acidic environment within vesicles. 3) activation of toxin due to a conformational change controlled by the acidic environment, followed by translocation of the L chain portion of the toxin into the cytoplasm. 4) catalysis of Soluble N-ethylmaleimide sensitive factor attachment protein REceptor (SNARE) proteins. SNARE proteins regulate vesicular exocytosis of neurons. Proteolytic cleavage of SNARE proteins prevents the fusion of acetylcholine vesicles with the plasma membrane, resulting in blockade of neuromuscular transmission and muscle paralysis. Histological changes observed in muscles injected with Clostridium botulinum toxin include the conservation of anatomical contact with nerves without apparent loss of motor axons and the accumulation of small synaptic vesicles on the cytosolic side of the plasma membrane. During renewed neuromuscular transmission, electrical activity from nerves causes pre-synaptic and post-synaptic production of neurotropins that induce nerve sprouting around the motor end-plate resulting in the formation of new nerve fibers from a single motor axon. There is also enlargement of the motor end-plate and spreading of acetylcholinesterase and acetylcholine receptors in other areas of the muscle plasma membrane. During a second phase of repair there is total regeneration of the original motor end plates and the elimination of nerve sprouts.

In humans, BTA is currently used for the treatment of chronic anal fissures. One or two injections with toxin into the internal or external anal sphincter caused
relaxation of the anal canal and allowed healing of chronic anal fissures, presumably due to improved blood flow and decreased tension of the fissure edges.\textsuperscript{36} In one study, BTA injections into the internal anal sphincter produced 26\% and 29\% reduction in resting anal pressure one and two months after treatments, respectively.\textsuperscript{35} Injections with BTA in the external anal sphincter in humans produces relaxation of both the internal and external anal sphincters by local diffusion of the toxin.\textsuperscript{19} In our study, peak pressure, but not resting pressure, was significantly decreased in BTB treated horses. The internal anal sphincter is a continuation of the rectal wall and it is responsible for maintaining resting anal pressure whereas the external anal sphincter is a separate thick circular layer of striated muscle that is mainly responsible for voluntary peak anal contraction. It would therefore be expected that toxin injections in the large external anal sphincter of horses would primarily affect voluntary (peak) anal pressures and not resting pressures. Alternatively, our rectal probe may have not been sensitive enough to detect subtle changes in resting pressures.

Doses of 2,500 to 10,000 units of BTB are currently recommended as an effective and safe treatment for cervical dystonia in humans. The most common side effects are dry mouth (3-34\% of cases) and dysphagia (16-25\% of cases.) Side effects are the result of local diffusion of the toxin, and they are more common when higher doses of toxin are used.\textsuperscript{22} In our study, 2,500 units of BTB injected in the external anal sphincter of one horse produced clinical signs of botulism, including generalized weakness, depression, and dysphagia. Lack of toxic side effects with 500, 1000, and 1500 units of toxin suggested that these amounts are safe for injection at two sites in a muscle. Ingestion of Clostridium botulinum spores or pre-formed toxin causes the clinical syndrome of
‘botulism’ in horses, which is characterized by generalized muscle weakness; low head carriage; mild depression; slowness drinking or eating; dysphagia; decreased tongue tone, decreased eyelid and tail tone; mydriasis; recumbency; colic; and in severe cases death due to respiratory paralysis or complications from prolonged recumbency.\textsuperscript{14,15} The rate of progression of clinical signs after ingestion of contaminated feed depends on the dose of toxin and the disease is usually fatal unless treated promptly with the specific antitoxin. Horses are most commonly affected by type B, and occasionally by type C or type A. An effective toxoid vaccine is available for \textit{C. botulinum} type B in horses.\textsuperscript{53-54} It is unknown if horses would respond to local injections of BTB after vaccination.

Injection of BTB in the external anal sphincter of horses caused a reduction in peak anal pressure in all horses (500-2500U) compared to control animals. Maximal effect of the toxin was observed within the first 15 days after injections followed by a slow return to baseline over 168 days. Onset of muscle relaxation after local injection with BTB for treatment of human cervical dystonia occurs within a few days to 1 week and peak effect is observed within 2 weeks. Individual variation was also observed in experiment 2, with the horse injected with 1000U of BTB having a greater per cent drop in peak anal pressure compared to the horse receiving 1500U. This was an unexpected finding because a similar or greater reduction of muscle function would be expected with increasing doses of BTB. The significant change in three horses injected with 1000 units of BTB suggests that this dose has a local effect for approximately 3 months. This duration is similar to duration in human muscle relaxation, which may last for 3 to 4 months after injections.\textsuperscript{30}
This study was initiated with the idea that BTB could be injected into the external anal sphincter of mares prior to surgical repair of perineal lacerations. Dehiscence of perineal incisions due to pressure from accumulation of feces in the rectum is a commonly reported complication after surgical repair.\textsuperscript{47-48} The decreased muscle tone after BTB injection may allow repair of the anal sphincter at the same time the rectovaginal shelf and perineal body are repaired rather than using a two stage technique.\textsuperscript{47} Since peak effect of BTB in horses was observed 15 days after injections, we recommend treatment with BTB for the repair of perineal lacerations least 15 days before surgery. In this study, a single injection of BTB at the 12 o’clock position caused a reduction of peak anal pressures but the ventral portion of the anal canal retained normal muscle thickness, which indicated lack of diffusion of the toxin in the anal sphincter. Injections at the 10 and 2 o’clock positions administered to achieve diffuse relaxation of the entire anal canal did not produce relaxation of the most ventral portion of the anal sphincter. Because perineal lacerations occur at the ventral portion of the anal canal, we recommend injecting equal amounts of BTB at four equidistant sites in the external anal sphincter.

In summary, local injections of botulinum toxin type-B in the external anal sphincter of horses caused reversible reduction of peak anal pressures. Systemic side effects were observed in one horse, which suggested a narrow dosage range to avoid toxicity. Further research to test the effects of botulinum toxin in clinical cases is needed to determine the full potential of this treatment modality. Conditions such as contracted tendons, laminitis, club foot conformation, spasm of back muscles, cribbing, perineal
lacerations in mares, and supraglenoid tuberosity fractures may benefit from transient decrease of muscle function after local botulinum toxin injections in horses.
THESIS CONCLUSIONS

Results of our study present the following important conclusions:

1. Botulinum toxin type B (BTB) injection in the external anal sphincter of horses produces prolonged relaxation of the anal canal.

2. Horses can be extremely sensitive to BTB and clinical signs of botulinum can be observed several days after local injection.

3. Injections of BTB in horses in the external anal sphincter may be useful for the treatment of perineal lacerations in mares and for conditions that benefit from transient reduction of neuromuscular activity, such as contracted tendons, laminitis, club foot conformation, spasm of back muscles, cribbing, and supraglenoid tuberosity fractures.


Vita

David Adam-Castrillo is the second child of three, born in Madrid on April 26 1971. His two bothers and he were raised in a small farm in Valdemorillo, a small village in the Sierra del Norte of Madrid. At a young age he was very interested in the local wild animals. He brought home snakes, lizards, frogs, birds, rats, scorpions, etc… His parents were very supportive and helped him build terrariums to keep the little creatures. As a teenager, he developed a strong interest in riding and fixing motorcycles. His father Javier was an engineer and taught him the basic principles of combustion engines. He learned to fix his own old motorcycles, even if it meant long night hours. When David was fifteen, his parents bought their first horse, and he soon became interested in horses. He became an avid rider with devotion for show jumping. He learned to shoe his own horses and spent a lot of time with the local veterinarians. During the summer months of 1987 William Manley, the family’s veterinarian, sent him to visit friends in Maryland and David learned about the ‘American way of life.’ He had a wonderful time with Sue and Dr. Larry Cushing, so much that his mother Concha became very jealous of ‘Papá y Mamá USA.’ During one of the farm calls he visited the New Bolton Center of the University of Pennsylvania and thereafter his dream was to study veterinary medicine in the US. After high school David spend one year at the Westmoreland Davis Equestrian Institute of Leesburg, Virginia, a training center for riding instructors. He was awarded the trophy to the best graduate of the Class of 1990. Upon returning to Spain, he enrolled to study at the Center for International Studies, an American university located in Madrid. Tuition fees were too high for David’s parents, and he decided to accept a job offer as a groom for a Spanish international rider. He traveled throughout Europe with the
Spanish Olympic Team of show jumping. After five months, the Center for International Studies offered a partial scholarship and David returned to study. He also worked as a blacksmith in the evenings and weekends. After two years he earned a full tuition scholarship at Boston’s Suffolk University where he later completed a Bachelor’s degree in Biology. He was admitted at the University of Pennsylvania’s veterinary school in 1994. With unbelievable effort his parents supported the high cost of the education and David obtained his veterinary degree in 1998. He was awarded the Sports Medicine and Imaging Award from New Bolton Center. His first job as an intern veterinarian was at The Mid-Atlantic Equine Medical Center in New Jersey. After the internship he worked at an ambulatory practice during the summer of 1999 and met his wife Miriam. In the fall of 1999 David was offered a position to work as a clinical fellow at Oregon State University. This job prepared him well to match the surgery residency at the Marion duPont Scott Equine Medical Center of Virginia Tech in 2000. David learned about botulinum toxin in Oregon through a veterinarian who suffered a nerve injury and was being treated with Botox to balance the activity of the flexor and extensor muscles of his hand. His idea of using botulinum toxin in horses developed while evaluating a horse with chronic contracture of flexor tendons. After graduation, David will move to Pennsylvania to work in equine private practice.