ORAL LD$_{50}$ OF BOTULINUM TOXIN SEROTYPE A IN GUINEA PIGS

A thesis submitted in partial fulfillment
of the requirements for the degree of
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By

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2007
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As a disease, botulism is a neuroparalytic illness resulting from the action of a potent neurotoxin produced by *C. Botulinum*. Of the seven distinct *C. Botulinum* neurotoxin serotypes: A, B, C, D, E, F, and G, only serotypes A, B, E and F cause human disease. The duration of action of the seven toxin serotypes varies with serotype A having the most sustained action (i.e. months vs. days in serotype E). This study was performed due to recently identified critical gaps in our food safety procedures. The goal of this study was to assess whether fresh 2% milk provided protective qualities to Botulinum toxin serotype A (BoNT/A) against degradation in the gastrointestinal tract. If protected this would lead to half the members of the tested population being killed with lower doses compared to toxin delivered in a conventional delivery vehicle (i.e. gel phosphate buffer). The model used for this study was adult male and female specific pathogen free Hartley guinea pigs. The pathophysiologic effects on guinea pigs following oral administration of BoNT/A was determined through daily clinical observations and utilization of telemetry implants allowing examination of changes in the mean, systolic, diastolic and pulse pressures, electrocardiogram (ECG), temperature, activity, respiratory rate, and heart rate. No difference was shown between the two treatment groups of 2% milk and gel phosphate buffer with respect to the time to first clinical sign or time to death. The LD$_{50}$ of BoNT/A in 2% milk was not significantly different from BoNT/A in gel phosphate buffer indicating that the milk did not provide protective qualities in combination with the toxin.
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INTRODUCTION

_Clostridium botulinum_ (first termed ‘botulus’, from the Latin word for sausage) is a spore forming gram positive, rod-shaped bacterium approximately 0.5-2.0µm in width and 1.6-22.0µm in length normally found in soil, water, and the intestinal tracts of humans and other animals. It was studied in the 1800s by Justinus Kerner, who noted several important observations about the bacteria and its toxins in his studies, including survival under anaerobic conditions, interruption of motor signal transmission, and lethality in small doses (Ting and Freiman, 2004).

As a disease, botulism is a neuroparalytic illness resulting from the action of a potent neurotoxin produced by _C. botulinum_. Common symptoms in humans include ptosis, dry mouth, fatigue, muscle weakness, constipation, ataxia, diarrhea, paresthesia, vomiting, reduced or failed reflexes, double/blurred vision, dysarthria and dysphagia. Paralysis from the disease presents as an acute, symmetrical descending paralysis accompanied by multiple cranial nerve palsies [(Shapiro et al., 1998) and (Amon et al., 2001)]. Deaths most commonly occur from respiratory failure.

Of the seven distinct _C. botulinum_ neurotoxin serotypes: A, B, C, D, E, F, and G, only serotypes A, B, E and F cause disease in humans. Each distinct serotype is produced by a distinct strain of _C. botulinum_ (Paiva et al., 1993). These neurotoxins are antigenically distinct but comparable in basic structure. Each toxigenic Clostridia produces a relatively inactive polypeptide of 150 kDa activated by proteases activated by the organism itself or gastrointestinal enzymes of the affected animal (US Department of Health and Human Services, 1998). The duration of action of the seven toxin serotypes varies, although serotype A is known to have the most sustained action (Foran et al., 2003; Keller and Neale, 2001; Raciborska and Charlton, 1999). In comparison, Serotype A can cause paralysis for many months whereas Serotype E
(both acting on SNAP-25) blockade lasts for brief periods. As described in a recent article on an outbreak of botulism in Italy, the duration of hospitalization of patients infected with Botulinum serotype A ranged from 37 to 78 days (Aureli et al., 2000).

Botulinum toxin is released from bacteria as part of a noncovalent multimeric complex. The auxiliary proteins that are best characterized are hemagglutinins (HA) and a nontoxin, nonhemagglutinin (NTNH). This complex plays no critical role at the site of toxin action, but is critical to the process of oral poisoning by providing resistance of the toxin to the low stomach pH and proteolytic enzymes in the gastrointestinal system allowing delivery of the toxin to vulnerable cells. (Simpson, 2004)

Active neurotoxin consists of a 100 kDa heavy chain and a 50 kDa light chain linked by a disulfide bond. Crystalline structures of Botulinum neurotoxin indicate that the heavy chain consists of a binding carboxyl domain and a translocation amino domain, while the light chain contains a catalytic domain (Shukla and Sharma, 2005). The likely mechanism allowing the toxin to cross the membrane barriers in the lumen of the gut or airway is the binding to the apical surface of epithelial cells leading to receptor-mediated endocytosis and/or transcytosis leading to delivery to the basolateral surface of cells (Maksymowych and Simpson, 1998).

In order for the toxin to function, the following chain of events must occur: 1) the carboxyl domain of the heavy chain binds to membrane receptors on the neuronal surface by lock and key with little conformational change to the neurotoxin; 2) low affinity receptor binding brings the neurotoxin in close proximity to bind to a second high affinity receptor allowing endocytosis of the neurotoxin; 3) the heavy chain amino domain forms a channel mediating translocation and the release of the light chain domain across the endosome membrane into the terminal cytoplasm (Shukla and Sharma, 2005); 4) a conformational change induced by the
acidic pH of the endosome allows the insertion of the neurotoxin into the endosomal membrane mediated by dissociation of the amino terminus of the heavy chain from the light chains; and 5) the catalytically active domain of the light chain is translocated from the lumen side to the cytosolic side of the endosome. This occurs through the reduction of the disulfide bond that links the light chain to the heavy chain, allowing for the movement of the light chain into a more neutral environment (Simpson, 2004). Enzymatic cleavage of the protein components of the neuroexocytosis apparatus by the light chain prevents the release of the neurotransmitter acetylcholine from synaptic terminals of the motor neurons at the neuromuscular junction (US Department of Health and Human Services, 1998) causing paresis in the target tissue. Botulinum neurotoxin serotype A targets the SNAP-25 (soluble N-ethylmaleimide-sensitive fusion attachment protein) of the SNARE (soluble NSF attachment protein receptors) complex. The SNARE complex is a group of proteins essential for the docking and fusion to the synaptic vesicles with the presynaptic membrane.

Six different forms of botulism exist. These include food-borne botulism (with approximately half of the cases in the United States being caused by neurotoxin A (Shapiro et al., 1998), wound botulism, infant botulism, adult form of infant botulism, inadvertent systemic botulism and inhalation botulism (Arnon et al., 2001). The majority of human cases are infant botulism. This form is more prevalent due to a lack of competing gut flora in infants. Food-borne botulism is the second leading cause of botulism. Food-borne botulism, inadvertent systemic and inhalational botulism all involve toxicity due to the exposure to preformed toxin (US Department of Health and Human Services, 1998).
Figure 1. Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell. B, Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. SNARE indicates soluble NSF-attachment protein receptor; NSF, N-ethylmaleimide-sensitive fusion protein; and SNAP-25, synaptosomal-associated protein of 25 kd.

Food borne botulism is caused by vegetative cells of *C. botulinum* that release preformed toxins in contaminated food. The main factors for growth of *C. botulinum* in food are: low temperature, low pH, low water activity, redox potential, food preservatives and competing microorganisms (US Department of Health and Human Services, 1998). Proteolytic strains of the bacteria grow optimally at 40°C and a minimum pH of 4.6-4.8. The time to onset of the 
Disease following a food borne exposure varies from several hours to 10 days, but most commonly from 18-36 hours. Initial symptoms of food borne botulism are: nausea, vomiting, abdominal cramps and diarrhea. Symptoms can progress into neurological problems such as blurred vision and dry mouth with progression into descending paralysis (Shapiro et al., 1998). Although some of the poisoned junctions may regain function, evidence of neuromuscular deficits can persist for months (Sugiyama, 1980). Electrophysiological evidence (supported by histological evidence) suggests a correlation between recovery of muscle function and development of new neuromuscular junctions (Sugiyama, 1980).

Deliberate release of Botulinum toxin in a civilian population food supply would cause substantial disruption and distress. The Botulinum toxin is extremely potent, lethal and is easily produced and transported. Infection with the toxin causes a need for prolonged intensive care (Arnon et al., 2001). Between 90% and 100% of cases reported are admitted to the hospital, with care lasting from one week to four or more weeks. (Przybylska, 1994) For this reason, the study of C. botulinum in food sources is imperative to close gaps that might cause confusion or question in the event of a bioterrorist attack. In addition, examination of the toxins stability in different food matrices that could serve as a potential target for contamination requires further investigation. It has been shown previously that fatty and proteinaceous foods increase potency of orally administered botulinum toxin. The exception being skim milk powder that showed fewer deaths with toxin relative to the controls. (Lamanna and MEYERS, 1960) Currently, it is thought that as many as 17 countries are suspected to include or to be developing biological agents in their offensive weapons programs (Shapiro et al., 1998).

This study was performed to address recently identified critical gaps at a Bioshield Scenario Planning Workshop pertaining to the determination of estimates of the number of
people that could be affected through the introduction of Botulinum toxin serotype A into a bulk milk source. A shipment of milk leaving a processing facility can contain between 4700 and 5400 gallons of pasteurized milk. Assuming that a family of 4 buys one gallon of milk the possible number of people affected by the contamination would be 18,800 – 21,600 individuals. Currently, the LD$_{50}$ dose of Botulinum toxin serotype A has been determined when delivered alone; however, no assessment of toxicity has been made for the toxin when delivered in milk. The affects of the milk on the toxicity are in question. The objective of the proposed research is to determine the oral LD$_{50}$ for BoNT/A in adult guinea pigs when administered in 2% milk via orogastric gavage. Perhaps the oral LD$_{50}$ for BoNT/A will be lowered by the toxin being present in milk, increasing the pH of the stomach allowing the toxin to pass more easily to the intestine. The toxin would have a more stable environment for protection and be more available for uptake in the intestines. The relative toxicity will be compared with that of the LD$_{50}$ dose of BoNT/A when delivered in gel phosphate buffer.
PURPOSE

Hypothesis

The administration of Botulinum neurotoxin serotype A (BoNT/A) in fresh 2% pasteurized milk will lower the LD$_{50}$ in the adult guinea pig in comparison to the normal gel phosphate buffer used in toxicity assays.

Specific Aims

The intent of this study was to address recently identified critical gaps pertaining to the determination of estimates of the number of people that could be affected through the introduction of Botulinum toxin serotype A into a bulk milk source.

1. To assess whether fresh 2% milk provides protective qualities for the toxin against degradation in the gastrointestinal tract (e.g. stomach) lowering LD$_{50}$ values in comparison to toxin delivered in conventional gel phosphate buffer.

To view the pathophysiologic effects on guinea pigs after the oral administration of BoNT/A through clinical observations and utilization of telemetry implants allowing examination of changes in the mean, systolic, diastolic and pulse pressures, ECG, temperature, activity, respiratory rate, QA interval, and heart rate.
METHODS

Test System

The model used for this study was adult male and female specific pathogen free Hartley guinea pigs (caviae porcellus). 110 guinea pigs (7-11 weeks of age) all weighing approximately 500 grams, randomized into groups containing approximately equal numbers of males and females were placed on study. Due to cost and availability, two guinea pigs (16-18 weeks of age) received as gratis animals were also used on study. Only guinea pigs free of obvious clinical signs of disease or malformations were utilized. Animal dosing and tissue collection were performed at Battelle Memorial Institute/Battelle Biomedical Research Center.

Prior to toxin dosing, each animal was conditioned with 1 ml of freshly pasteurized 2% milk (Kroger Co., Tamarack Farms, Newark, Ohio) or control material gel phosphate buffer (Battelle) dependent on their study group. Dose was set to be 3.25ml (compared to the human stomach holding a one cup serving of milk) However due to the guinea pigs not being able to manage the volume – it was determined that animals would be dosed with 1 mL. During conditioning and toxin dosing, the milk and gel phosphate buffer were administered via orogastric gavage. For this study, “orogastric gavage” is defined as the insertion of a 2” stainless steel 18 gauge needle with a ball tip inserted through the mouth and esophagus into the stomach. Each animal was sedated prior to dosing with 0.2 ml of Xylazine\textsuperscript{TM}. Administered challenge doses contained various levels of Botulinum neurotoxin serotype A.
**BoNT/A in 2% milk LD₅₀ determination**

The LD₅₀ of the Botulinum neurotoxin serotype A in 2% milk was determined using a 3 phase approach via orogastric intubation challenges and compared to the control material, Botulinum neurotoxin serotype A in gel phosphate buffer. Doses for later phases were selected based on the cumulative results of earlier phases. The cumulative results from all three phases were used to determine the LD₅₀ dose of Botulinum neurotoxin serotype A in 2% milk and PB and to determine any differences between the 2% milk and PB groups. Botulinum neurotoxin serotype A characterized with a known mouse intraperitoneal lethal dose 50/ml (MIPLD₅₀) was used to prepare the required Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer dosing solutions.

**Determination of MIPLD₅₀ Units (potency)**

The determination of the MIPLD₅₀ of the Botulinum neurotoxin serotype A was done by a mouse potency assay prior to the start of the study. A MIPLD₅₀ Unit is the quantity of toxin which when injected intraperitoneally (IP) into mice, is expected to cause the death of 50% of the mice within ninety-six hours. Botulinum toxins are quantitated by their potency or activity, not weight and one unit of toxin is the LD₅₀. The process to characterize the BoNT/A used in this study included was done by these assay steps: 1) Obtaining mice weighing 18-22 grams, 2) setting up multiple graded dilutions (in sterile gel phosphate buffer) of the Botulinum toxin, 3) inject groups of mice IP with approximately 0.5 mL of toxin at each concentration, where the number of mice per group was based on the required precision, 4) record the number of dead mice as well as live mice per group daily for a period of four days (i.e. 96 ± 2 hr from the time of IP injection), and 5) assign the toxin stock vials the MIPLD₅₀ dose based on the number of mouse deaths in MIPLD₅₀ units per mL using probit analysis. At a minimum, one experimental
group must have 50 percent or greater survival and one group, 50 percent or greater mortality for the experimental results to be valid (i.e., the experimental curve must touch or span the 50 percent mortality level).

**Preparation of BoNT/A-M and BoNT/A-PB**

Prior to the day of oral dosing, the appropriate volume of Botulinum neurotoxin serotype A was added and mixed with fresh 2% milk or gel phosphate buffer at 4°C to produce a 1.0 ml total volume/animal dose. The Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer was kept at 4°C for ~16-24 hours prior to use for oral dosing. The dosing solution was kept overnight to establish the amount of time that the product could be sitting on store shelves before the consumer purchases the contaminated product. Due to volume problems (i.e. animals unable to tolerate larger volumes via oral gavage), the originally desired dosing volume per animal [based on the percent volume (~25%) occupied by a one cup (237 ml) serving of milk of an adult human stomach (capacity of ~940 ml)] of 3.25 ml could not be used and the volume was decreased to 1.0 ml.

The fresh 2% milk obtained from Tamarack Farms, Kroger Company in Newark OH used in this study was within 12 hours of the pasteurization process when toxin was added. The reason for using milk less than 12 hours post pasteurization was to make the situation as close to an incidence of milk being contaminated prior to shipment to a grocery store. To verify the time requirements, the processing and time of the pasteurization was obtained from the supplier of the 2% milk (Table 1).
Table 1. Pasteurization Processing

<table>
<thead>
<tr>
<th>Date</th>
<th>Start Time</th>
<th>End Time</th>
<th>Temp (°F)</th>
<th>Cooled To (°F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/11/2005</td>
<td>0645</td>
<td>0820</td>
<td>176</td>
<td>36.5</td>
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</tr>
<tr>
<td>11/1/2005</td>
<td>0445</td>
<td>0630</td>
<td>180.5</td>
<td>36.5</td>
</tr>
<tr>
<td>11/15/2005</td>
<td>0715</td>
<td>0850</td>
<td>179</td>
<td>36.5</td>
</tr>
</tbody>
</table>

Phase 1 Botulinum Serotype A Administration

Initially, fifty two guinea pigs were randomized into ten groups of 5 animals and one group of 2 animals for dose day (Table 2). Due to limitations in the telemetry system, phase 1 was divided into two phases for oral dosing. However both phases were performed in the same manner and will be combined in the phase 1 methods. On Study Day 0, each animal was lightly sedated with 0.2 mL of Xylazine™ (100 mg/mL) prior to oral dosing. Doses were delivered via an 18-gauge 2-inch stainless steel feeding needle with a 2.4mm ball tip. Doses were given to all groups except the control group as one of five MIPLD₅₀ dose levels of Botulinum serotype A diluted to desired levels in 1 mL of fresh 2% milk (groups 1-5) or gel phosphate buffer (groups 6-10) (Table 2). Group 1 consisted of 5 guinea pigs receiving a 2.9x10² MIPLD₅₀ oral dose of Botulinum serotype A, group 2 consisted of 5 guinea pigs receiving a 9.4x10² MIPLD₅₀ oral dose of Botulinum serotype A, group 3 consisted of 5 guinea pigs receiving a 3.0x10³ MIPLD₅₀ oral dose of Botulinum serotype A, group 4 consisted of 5 guinea pigs that received a 9.6x10³ MIPLD₅₀ oral dose of Botulinum serotype A, and group 5 consisted of 5 guinea pigs that received a 3.1x10⁴ MIPLD₅₀ oral dose of Botulinum serotype A, all delivered in 2% milk. Groups 6-10 received the same dose level corresponding to Groups 1-5, respectively, with the Botulinum serotype A only being delivered in gel phosphate buffer. Group 11 consisted of 2 animals and received 1 mL of 2% milk (no toxin) as the control group.
Table 2. Phase 1 – Treatment Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Guinea Pigs/Group</th>
<th>Phase</th>
<th>~Dose (MIPLD$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>$2.9 \times 10^{2}$</td>
</tr>
<tr>
<td>Milk</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>$9.4 \times 10^{2}$</td>
</tr>
<tr>
<td>Milk</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>$3.0 \times 10^{3}$</td>
</tr>
<tr>
<td>Milk</td>
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<td>5</td>
<td>1</td>
<td>$9.6 \times 10^{3}$</td>
</tr>
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<td>Milk</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>$3.1 \times 10^{4}$</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>$2.9 \times 10^{2}$</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
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<td>5</td>
<td>1</td>
<td>$9.4 \times 10^{2}$</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
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<td>5</td>
<td>1</td>
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</tr>
<tr>
<td>Phosphate Buffer</td>
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<td>5</td>
<td>1</td>
<td>$9.6 \times 10^{3}$</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
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<td>5</td>
<td>1</td>
<td>$3.1 \times 10^{4}$</td>
</tr>
<tr>
<td>Milk Control</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>NA</td>
</tr>
</tbody>
</table>

Phase 2 Botulinum Serotype A Administration

Thirty one guinea pigs were randomized into five groups of 5 animals, one group of 4 animals, and one group of 2 animals for day of dosing. Two females originally placed in group 2 were replaced prior to challenge with two females from group 4. Only one extra animal was able to be replaced back into group 4. Oral dosing (performed as previously described) began on Study Day 0 with each animal being sedated with 0.2 mL of Xylazine™ (100 mg/mL).

As seen in Table 3 group 15 consisted of 4 guinea pigs due to the replacement of animals in group 13. The replacement of animals from a gel phosphate buffer group to a milk group was based on the number of animals being more important in the group of study interest. The milk group is the basis for the whole study, whereas the gel phosphate group was used as a positive control. Group 18 was dosed with 2% milk (no toxin) as a control group.
Table 3. Phase 2 – Treatment Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Guinea Pigs/Group</th>
<th>Phase</th>
<th>~Dose (MIPLD50s)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>12</td>
<td>5</td>
<td>2</td>
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</tr>
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<td>Milk</td>
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<td>5</td>
<td>2</td>
<td>1.6 x 10^2</td>
</tr>
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<td>Milk</td>
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<td>5</td>
<td>2</td>
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<td>5.0 x 10^1</td>
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<td>5.2 x 10^2</td>
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<tr>
<td>Milk Control</td>
<td>18</td>
<td>2</td>
<td>2</td>
<td>NA</td>
</tr>
</tbody>
</table>

Phase 3 Botulinum Serotype A Administration

Twenty six guinea pigs were randomized into four groups of 6 animals and one group of 2 control animals for day of dosing. One additional animal was added to each group in Phase 3 due to availability. Animals were anesthetized and dosed orally as previously described.

Table 4. Phase 3 – Treatment Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Guinea Pigs/Group</th>
<th>Phase</th>
<th>~Dose (MIPLD50s)</th>
</tr>
</thead>
<tbody>
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<td>6</td>
<td>3</td>
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</tr>
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<td>6</td>
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</tr>
<tr>
<td>Phosphate Buffer</td>
<td>22</td>
<td>6</td>
<td>3</td>
<td>3.6 x 10^2</td>
</tr>
<tr>
<td>Milk Control</td>
<td>23</td>
<td>2</td>
<td>3</td>
<td>NA</td>
</tr>
</tbody>
</table>
Telemetry Monitoring

Telemetry readings were monitored and recorded once every 5 minutes for 30 seconds during baseline and post-challenge observation periods via a transponder (C50-PXT, DSI, St Paul, MN). C50-PXT transponders were surgically implanted in the peritoneal cavity of each guinea pig at Charles River Laboratories. Each transponder consisted of two biopotential leads (used to record ECG waveforms) and a pressure lead (used to record mean, systolic, diastolic and pulse pressure values). Animal racks were set up with an individual receiver for each animal cage, where each animal was caged individually. The receivers collected and transmitted data through a data exchange matrix to a computer for data storage, viewing, and analysis. RPC-1 small animal and RMC-1 large animal receivers (both compatible with the C50-PXT transponder) were both used in the collection of data. Animals were staggered on the racks to prevent any possible crosstalk between the receivers. The telemetry system was configured before each phase of the study. The configuration identified the animals for each phase and their respective position in the collection process. Baseline values were recorded beginning two days prior to Study Day 0 for each phase. Depending on the time added to study, replacement animals did not all have the full two day baseline.

Clinical Observations

Post-dosing, guinea pigs were observed three times daily until termination (day 6) for clinical signs of illness and survivability due to Botulinum. All observations were done during the normal working day, between the hours of 0600 and 1700. Deaths were recorded to the nearest day and euthanasia to the nearest minute. Guinea pigs were observed for the following, but not limited to, signs of illness: respiratory distress, not eating, dehydration, paralysis, eyelid closure, rough hair coat, activity, moribund and death.
Necropsy and Histopathology

Gross necropsies were performed throughout the 7 day observation period on all animals found dead, euthanized due to illness, and on all animals surviving the 7 day post-dosing period following euthanasia. All tissues taken were fixed in formalin and sent to EPL Archives (Virginia) for archival; however, histopathology was not performed.

Sample Size

The sample size of ~5 guinea pigs per dose-response curve was sufficient that the half width of the 95 percent confidence interval for the LD\textsubscript{50} was 30 percent or less. The half width of the confidence interval depended on variability in the estimated LD\textsubscript{50}, which was a function of the number of animals dosed, the slope of the dose-response curve, and the allocation of animals to the dose-response relationship.

Statistical Methods

Probit dose-response models were fit using the SAS\textsuperscript{®} (version 9.1) PROBIT procedure. In order to determine if the two groups had the same dose-response relationship, separate models were fit for each group. A likelihood ratio test was used to determine if the common slope model adequately fit the data. Estimates for the LD\textsubscript{50} and confidence intervals were calculated from the probit dose-response model.

The LD\textsubscript{50} ratio between the two treatment groups was computed in order to assess the toxicity of Botulinum neurotoxin serotype A in 2% milk in comparison to gel phosphate buffer. For the LD\textsubscript{50} ratio, a 95 percent confidence interval was calculated using Fieller’s theorem. Refer to Finney (1971) for a more complete description of Fieller's theorem. A statistical hypothesis test was conducted by comparing the LD\textsubscript{50} ratio to one (unity) to determine whether the estimated LD\textsubscript{50}s were significantly different from each other.
For the analysis of time to death and time to clinical signs, a nonparametric survival analysis procedure was used that incorporated right-censoring indicators for animals that survived through time point 18 (one animal was observed for 19 time points). Since days to death or clinical signs were a more informative measure than time points, the time points were converted into days. There were three time points per day (morning, mid-day, and afternoon), except for the first day where dosing was administered in the morning. To convert the time points after dosing to days, the time points were simply divided by three. Thus, if an animal was observed at the third time point (morning of day after dose) with some clinical sign then the days to clinical sign would be 1. Descriptive statistics (mean and standard errors) of the days to death and clinical signs were tabulated for each group using the Kaplan-Meier product limit method. The LIFETEST procedure of SAS® (version 9.1) was used for this analysis. In order to adjust for the different doses given in determining the effect of the group on days to death, Cox proportional hazards modeling was used. The TPHREG procedure of SAS® (version 9.1) was used for this analysis. The log base 10 of the dose was used for all analyses. The Cox model included the treatment group, the dose effect and the interaction between treatment group and dose.

**Electrocardiographic Assessment**

ECG waveform tracings were generated prior to the dosing and as scheduled throughout the observation periods (i.e. 30 seconds every 5 minutes). The ECG waveform strips were qualitatively assessed for rhythm and morphology alterations by a board certified veterinary cardiologist. ECG waveforms from the final ~24 hours of life were viewed at approximately 1.5 second intervals each hour to show morphology and approximately 9 second intervals each hour to show rhythm.
MATERIALS

Test Material

The test material used in this study was Botulinum neurotoxin serotype A (Etiologic ID# CBA102) (provided by Metabiologics, Inc, Madison, WI) diluted to the desired toxin levels in 2% milk or gel phosphate buffer prepared by Battelle (West Jefferson, OH). All dilutions were based on the stock concentration of toxin of $2 \times 10^5 \text{MIPLD}_{50}/\text{mL}$. The stock concentration was previously determined by mouse potency assays where multiple graded dilutions of the Botulinum toxin were dosed intraperitoneally into mice.

Control Material

The control materials used in this study were fresh 2% pasteurized milk provided by Tamarack Farms of the Kroger Company or gel phosphate buffer formulated at Battelle. The 2% milk was obtained from the supplier the same morning the toxin was to be added.
RESULTS

Clinical Observations

A secondary goal of this study was to establish time to death and time to onset of clinical signs, through clinical observations and the use of telemetry. Following dosing of the animals, the clinical signs observed included dyspnea, dehydration, lethargy, rough hair coat, not eating, paresis, and paralysis. Signs were most commonly observed in animals receiving lower dose levels of toxin. Animals receiving higher doses of Botulinum neurotoxin serotype A in phase 1 were commonly found dead within the first 24 hours post-dosing and no signs were observed. However, when compared to data obtained from the telemetry monitoring, it is likely that clinical signs would have been noted if more frequent observations were performed. The most common clinical observations were dyspnea, lethargy, and rough hair coat. Control animals showed no clinical signs of disease and were all normal at the conclusion of each phase of the study. In addition, animals that were dosed in the later phases (i.e. lower toxin doses) also commonly showed signs of paresis and paralysis along with increasing severity of dyspnea up to the time of death. As compared to phase 1, phases 2 and 3 showed disease with animals in the higher dose groups exhibiting prolonged clinical signs through the end of each phase. Each phase showed that animals dosed with a higher LD$_{50}$ fewer signs were exhibited. In phases 2 and 3, signs were not seen until approximately forty-five to forty-eight hours post dosing. This shows that the LD$_{50}$ was being narrowed and Botulinum has a delayed onset at lower doses. Compared to the last two phases, phase 1 animals were found dead and exhibiting signs at less than twenty-four hours.

Probit dose-response models were fit using the SAS® (version 9.1) PROBIT procedure. In order to determine if the two groups had the same dose-response relationship, separate models were fit for each group. Then an additional model was fit in which the two groups were forced
to have a common slope but individual intercepts, resulting in two parallel curves. A likelihood ratio test was used to determine if the common slope model adequately fit the data. Estimates for the LD50 and confidence intervals were calculated from the probit dose-response model.

The p-values indicate that there is no difference between the two treatment groups of 2% milk and gel phosphate buffer with respect to the time to first clinical sign. The Cox proportional hazards model for the time to first non-normal clinical sign indicated that there was no difference between the 2% milk group and the gel phosphate buffer group (p-value=0.6502) but that dose did affect the time to onset (p-value<0.0001). This indicates that the dose-response for time to onset of clinical signs was not significantly different between treatment groups. An exception to the dose-response trend occurred in the milk group at the 160 MIPLD50 dose level, where the lethality rate was greater than that observed at higher doses.

Table 5. Descriptive Statistics (Mean and Standard Errors) For Days to Death and Onset of Clinical Signs and Log-Ranked P-Values for Testing Group Differences

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total # of Animals</th>
<th>Predicted Mean (S.E.) Days to Death</th>
<th>Overall Group P-Value (Death)</th>
<th>Predicted Mean (S.E.) Days to Clinical Sign</th>
<th>Overall Group P-Value (Clinical Sign)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>52</td>
<td>3.72 (0.304)</td>
<td>0.8924</td>
<td>2.69 (0.246)</td>
<td>0.9376</td>
</tr>
<tr>
<td>PB</td>
<td>51</td>
<td>3.82 (0.307)</td>
<td></td>
<td>2.93 (0.282)</td>
<td></td>
</tr>
</tbody>
</table>
Mortality

A primary goal of this study was to assess the toxicity of Botulinum neurotoxin serotype A when administered via orogastric gavage and delivered in 2% pasteurized milk or gel phosphate buffer. Following dosing in phase 1, all animals in groups 3-5 (Botulinum serotype A in 2% milk), and 7-10 (Botulinum serotype A in gel phosphate buffer) died with an average group time to death of 1.0-2.0 days post-dosing. The average times to death, standard deviations, and the ranges are summarized in Table 6. There was a decreased mortality rate and extended average time to death in the two lower doses of toxin in milk (groups 1 and 2) and the lowest dose of toxin in gel phosphate buffer (group 6) (see Table 6). Generally, a dose-dependent mortality rate and average time to death was observed in all groups except controls and group 1 listed in Table 6, whether the toxin was diluted in 2% milk or gel phosphate buffer. Animals in group 11 that received 2% milk as the controls showed no mortality.

Based on the results of phase 1, where all animals succumbed to disease in all groups except for the low dose group and one animal from the second to low dose, dose levels of Botulinum neurotoxin serotype A in 2% milk or gel phosphate buffer were designed to bracket the lowest dose during that phase. Levels for phase 2 were therefore set above and below 2.9x10^2 MIPLD_{50}s. Within the phase, a dose-dependent mortality rate was seen in animals dosed with either Botulinum neurotoxin serotype A in 2% milk or gel phosphate buffer (Table 7). There were mortality rates of 0% (0/5), 60% (3/5), and 80% (4/5) for 2% milk groups 12 (low dose), 12 and 14 (high dose), respectively, in phase 2 (Figure 4). However in groups dosed with Botulinum neurotoxin serotype A in gel phosphate buffer lethality was observed only shown in group 17 (high dose) with a 4/5 mortality (Figure 5). No mortality was shown in the milk only control group 18.
Table 6. Mortality Data Phase 1

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Dose (MIPLD₅₀)</th>
<th>Number Dead</th>
<th>Avg time to death (days)</th>
<th>Std Dev time to death (days)</th>
<th>Min time to death (days)</th>
<th>Max time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ᵃ</td>
<td>2.9 x 10²</td>
<td>2 of 5</td>
<td>3.0</td>
<td>1.4</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>2ᵃ</td>
<td>9.4 x 10²</td>
<td>1 of 5</td>
<td>2.8</td>
<td>2.4</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>3ᵃ</td>
<td>3.0 x 10³</td>
<td>5 of 5</td>
<td>1.2</td>
<td>0.4</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>4ᵃ</td>
<td>9.6 x 10³</td>
<td>5 of 5</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5ᵃ</td>
<td>3.1 x 10⁴</td>
<td>5 of 5</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6ᵇ</td>
<td>2.9 x 10²</td>
<td>3 of 5</td>
<td>4.7</td>
<td>1.2</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>7ᵇ</td>
<td>9.4 x 10²</td>
<td>5 of 5</td>
<td>2.0</td>
<td>0.7</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>8ᵇ</td>
<td>3.0 x 10³</td>
<td>5 of 5</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9ᵇ</td>
<td>9.6 x 10³</td>
<td>5 of 5</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10ᵇ</td>
<td>3.1 x 10⁴</td>
<td>5 of 5</td>
<td>1.2</td>
<td>0.4</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>Milk Control</td>
<td>0 of 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

ᵃBoNT/A in milk
ᵇBoNT/A in gel phosphate buffer
Figure 2. Mortality Data Graph – Botulinum Neurotoxin Serotype A in Milk Phase 1

Figure 3. Mortality Data Graph – Botulinum Neurotoxin Serotype A in Gel Phosphate Buffer Phase 1
Due to the different mortality rates obtained in phase 2, doses chosen for phase 3 for Botulinum neurotoxin serotype A in 2% milk differed from those in gel phosphate buffer (Table 9). Doses for the Botulinum neurotoxin serotype A in milk were chosen to target the estimated \( \text{LD}_{40} \) \((1.9 \times 10^{2} \text{ MIPLD}_{50}s)\) and \( \text{LD}_{60} \) \((3.3 \times 10^{2} \text{ MIPLD}_{50}s)\) values (as well as bracket the predicted \( \text{LD}_{50} \) value) as predicted by the previous two phases. Doses for the Botulinum neurotoxin serotype A in gel phosphate buffer were chosen to target the estimated \( \text{LD}_{25} \) \((2.3 \times 10^{2} \text{ MIPLD}_{50}s)\) and \( \text{LD}_{65} \) \((3.6 \times 10^{2} \text{ MIPLD}_{50}s)\) values. Mortality rates in phase 3 (Table 8) were 50% (3/6) and 0% (0/6) for groups 19 and 20 receiving the Botulinum neurotoxin serotype A in milk and 67% (4/6), and 0% (0/6) for groups 21 and 22 receiving Botulinum neurotoxin serotype A in gel phosphate buffer (Fig 6 and Fig 7). Animals in the 2% milk only control group (phase 3, group 23) showed no mortality.

Overall, statistical evaluation using descriptive statistics for the days to death showed through calculated p-values that there was no difference between the two treatment groups with respect to the time to death. The Cox proportional hazards modeling also indicated that there was no difference in time to death between the 2% milk and gel phosphate buffer groups (p-value=0.4736), but that dose was highly significant (p-value<0.0001) in predicting the time to death, which was consistent with the probit analysis. The interaction between treatment group and dose was not significant and was dropped from the final model. This indicated that the dose-response for time to death was not significantly different between treatment groups. The summary of results for the \( \text{LD}_{50}s \) for both treatment groups milk and gel phosphate buffer are listed in Table 9.
Table 7. Mortality Data Phase 2

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Dose (MIPLD$_{50}$)</th>
<th>Number Dead</th>
<th>Avg time to death (days)</th>
<th>Std Dev time to death (days)</th>
<th>Min time to death (days)</th>
<th>Max time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12$^a$</td>
<td>5.0 x 10$^1$</td>
<td>0 of 5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13$^a$</td>
<td>1.6 x 10$^2$</td>
<td>3 of 5</td>
<td>4.0</td>
<td>1.0</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>14$^a$</td>
<td>5.2 x 10$^2$</td>
<td>4 of 5</td>
<td>3.0</td>
<td>1.4</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>15$^b$</td>
<td>5.0 x 10$^1$</td>
<td>0 of 4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16$^b$</td>
<td>1.6 x 10$^2$</td>
<td>0 of 5</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<td>17$^b$</td>
<td>5.2 x 10$^2$</td>
<td>4 of 5</td>
<td>3.0</td>
<td>0.8</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>18</td>
<td>Milk Control</td>
<td>0 of 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Figure 4. Mortality Data Graph – BoNT/A in Milk Phase 2
Figure 5. Mortality Data Graph – BoNT/A in Gel Phosphate Buffer Phase 2

Table 8. Mortality Data Phase 3

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Dose (MIPLD₅₀s)</th>
<th>Number Dead</th>
<th>Avg time to death (days)</th>
<th>Std Dev time to death (days)</th>
<th>Min time to death (days)</th>
<th>Max time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>1.9 x 10²</td>
<td>0 of 6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>20</td>
<td>3.3 x 10²</td>
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<td>0.6</td>
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<td>3.0</td>
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<tr>
<td>21</td>
<td>2.3 x 10²</td>
<td>0 of 6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>22</td>
<td>3.6 x 10²</td>
<td>4 of 6</td>
<td>3.8</td>
<td>1.3</td>
<td>2.0</td>
<td>5.0</td>
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<tr>
<td>23</td>
<td>Milk Control</td>
<td>0 of 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

ₐBoNT/A in milk
ₜBoNT/A in gel phosphate buffer
Stage 3 Milk Treated

Figure 6. Mortality Data Graph – BoNT/A in Milk Phase 3

Stage 3 Gel Phosphate Buffer Treated

Figure 7. Mortality Data Graph – BoNT/A in Gel Phosphate Buffer Phase 3
Table 9. Summary Of Statistical Results From Probit Analysis

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total No. Animals Tested</th>
<th>Probit Slope</th>
<th>Predicted LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Slope</td>
<td>P-value</td>
</tr>
<tr>
<td>Milk</td>
<td>52</td>
<td>2.42</td>
<td>0.0013</td>
</tr>
<tr>
<td>PB</td>
<td>51</td>
<td>6.53</td>
<td>0.0030</td>
</tr>
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</table>

Telemetry Trends

Pre- and post-dosing data collected on this study by the telemetry system included mean, systolic, diastolic, and pulse pressure, EKG, temperature, activity, respiratory rate, and heart rate. Several common trends were seen in the guinea pigs following dosing with Botulinum neurotoxin serotype A in 2% milk or gel phosphate buffer. These included the following: 1) increases in heart rate followed by a decrease just prior to death, 2) pulse pressures also had a tendency to rise just prior to the time of death, and 3) temperature showed a linear regression with the heart rate in that as one decreased near the point of death the other dropped at a similar rate. According to the telemetry data the best indicator of death appeared to be the pulse pressure. Times of death were determined by the time at which the pulse pressure dropped below zero. Animals that received lower doses of the Botulinum neurotoxin serotype A showed more gradual decreases in data compared to the high dose groups.

Although the study was ended on study day 7, the telemetry data showed that had the study been longer more animals could have succumbed to death. Animals that were observed visually as being normal or showing minimal clinical signs, showed decreasing temperature, blood pressures and slowing heart rates in the telemetry data.
Figure 8. Dose Response Relationship for Both Milk and Gel Phosphate Buffer
Telemetry Assessment

The results of the statistical analyses are presented in this section, separately by parameter and treatment group. All references to statistical significance are at the 0.05 level, unless specified otherwise in the methods section (e.g., comparisons of baseline averages among pairs of treatment groups were done at the 0.05/3=0.0167 level; pairwise comparisons to control group at a given post-dosing time point were done at the 0.05/10=0.005 level).

Body Temperature

For body temperature, significant dose effects started to present at 12 hours post-dosing and continued through the ANOVA tested 24 hours period for both Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer. The highest dose levels (31000 and 9600 MIPLD_{50}) showed significant differences from the control group. Low and medium dose levels did not show significant differences from the control group. The average body temperature also dropped significantly in the first two hours post-dosing, rose back to baseline temperature level after about 5 hours, and then rose slightly above the baseline level after ~6 hours for the Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer dose groups and the control group. For low and medium dose levels, average body temperatures remained slightly above the baseline levels throughout the 24 hour period for both Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer dose groups.
Figure 9. Graph is illustrating the average temperature for an animal being dosed in the Milk Dose Level 3.0x10^3 group. The temperature is shown dropping at the second event marker when the animal was anesthetized for dosing. At event marker three the temperature is ascending to normal as the animal recovers from anesthesia. At 60 hours plus the animal is succumbing to disease, representing by the drop in body temperature.

Figure 10. Graph is illustrating the average temperature for an animal being dosed in the Milk Dose Level 9.4x10^2 group. As seen in Figure 9, the temperature drops at time of anesthesia and recovers within ten hours of being dosed. This animal represents an animal that was normal through the study but is seen to have a decline in temperature towards the study end.
Figure 11. Graph is illustrating the average temperature for an animal in the Milk Control group. This animal shows the same drop in temperature at event marker one where anesthesia was given for dosing. At recovery, the animal shows a return to normal temperature throughout the remainder of the study.

For the high dose levels, average body temperatures dropped significantly after ~10-12 hours and continued to drop until all animals died (dose level 31000 MIPLD$_{50}$) or throughout the plotted 24 hour period. It appeared that the average body temperature dropped more rapidly for the high dose levels in Botulinum neurotoxin serotype A in gel phosphate buffer than in Botulinum neurotoxin serotype A in 2% milk. It should be noted that the significant drop in temperature near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.
Systolic Blood Pressure (SBP)

No significant dose effect on SBP in the first 24 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk. Botulinum neurotoxin serotype A in gel phosphate buffer, shows a significant dose effect on SBP and was present at 18 and 23 hours post-dosing. However, at 18 hours post-dosing, pairwise comparisons show that SBP in various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were not significantly different from SBP in the control group. At 23 hours post-dosing, SBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 9600 dose group (based on only one surviving animal) was significantly different from SBP in the control group. The average SBP dropped significantly in the first two hours post-dosing in all groups. The average SBP level rose in the next 2 hours (but did not reach the original baseline level) and remained relatively steady after 4 hours post-dosing. Generally, the control group had the lowest average SBP after 8 hours post-dosing. Before 8 hours post-dosing, many Botulinum neurotoxin serotype A in 2% milk and Botulinum neurotoxin serotype A in gel phosphate buffer dose levels had lower average SBP than the control group. In general, when comparing dose levels in groups, the high dose levels had higher average SBP than medium dose levels and medium dose levels had higher average SBP than low dose levels. It should be noted that the significant drop in SBP near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.
Figure 12. Graph is illustrating pressures for an animal dosed in the Milk Dose Level $5.2 \times 10^2$ group. In descending order the graph lines represent systolic pressure on the top, then mean pressure, diastolic and the bottom line represents the pulse pressure. This animal succumbed to disease within 48 hours of being dosed.

Figure 13. Graph is illustrating pressures for an animal dosed in the Milk Dose Level $9.4 \times 10^2$ group. In descending order the graph lines represent systolic pressure on the top, then mean pressure, diastolic and the bottom line represents the pulse pressure. This animal was found dead on the last day of study and is represented by a gradual decline in body pressures.
Figure 14. Graph is illustrating pressures for an animal dosed in the Milk Control group. In descending order the graph lines represent systolic pressure on the top, then mean pressure, diastolic and the bottom line represents the pulse pressure.

**Diastolic Blood Pressure (DBP)**

There was a significant dose effect on DBP at 24 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk and average DBP at the dose level of 31000 was significantly different from the control (based on only 1 surviving animal). The average DBP dropped significantly in the first two hours post-dosing for Botulinum neurotoxin serotype A in 2% milk group, rose back within an hour to the level of first hour immediately post-dosing, but remained below the original baseline level. The average DBP level remained relatively steady after 4 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk group. Generally, the control group had the lowest average DBP after 8 hours post-dosing. Between 5 and 17 hours post-dosing, the highest average DBP was found in Botulinum neurotoxin serotype A in 2% milk 3000 dose level.
Botulinum neurotoxin serotype A in gel phosphate buffer, shows a significant dose effect on DBP at 1 and 17 to 23 hours post-dosing. However, at the 1, 19, 20, 21, and 24 hours post-dosing, pairwise comparisons show that DBP in various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were not significantly different from DBP in the control group. At 17 hours post-dosing, DBP in Botulinum neurotoxin serotype A in gel phosphate buffer 31000 dose level (based on 3 animals) was significantly different from DBP in the control group; at the 18 hours post-dosing, DBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 31000 dose level (based on only 1 animal) was significantly different from DBP in the control group; and at 22 and 23 hours post-dosing, DBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 9600 dose level (based on only 1 animal) was significantly different from DBP in the control group. For the 50, 3000, and 9600 Botulinum neurotoxin serotype A in gel phosphate buffer dose levels, the average DBP rose above baseline in the first hour immediately post-dosing, dropped significantly in the 2nd hour post-dosing, and then rose back to a DBP level in line with other dose levels. The average DBP for the other Botulinum neurotoxin serotype A in gel phosphate buffer dose groups dropped continuously over the first two hours immediately post-dosing, rose and dropped back to the level of hour 2 post-dosing, and remained relatively steady after 6 hours post-dosing (but still below the original baseline level). Except for the Botulinum neurotoxin serotype A in gel phosphate buffer 230 dose group, the control group had lowest average SBP after 8 hours post-dosing. Among all Botulinum neurotoxin serotype A in gel phosphate buffer dose groups, the Botulinum neurotoxin serotype A in gel phosphate buffer 230 dose group had the lowest average DBP between 2 and 16 hours post-dosing.

It should be noted that the significant drop in DBP near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.
Mean Arterial Pressure (MBP)

There was no significant dose effect on MBP in the first 24 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk. The average MBP dropped significantly in the first two hours post-dosing for the Botulinum neurotoxin serotype A in 2% milk group, rose back within an hour to the level of first hour immediately post-dosing, and then dropped slightly to a relatively steady level after 4 hours post-dosing. The average MBP level remained relatively steady after 4 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk group. Generally, the control group had lowest average MBP after 8 hours post-dosing.

For Botulinum neurotoxin serotype A in gel phosphate buffer, a significant dose effect on MBP was present at 1, 18, and 23 hours post-dosing. However, at the first hour post-dosing, pairwise comparisons show that MBP in various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were not significantly different from the MBP in the control group. At 18 hours post-dosing, the MBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 31000 dose level (based on only one animal) was significantly different from MBP in the control group. At 23 hours post-dosing, the MBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 9600 dose level (based on only one animal) was significantly different from MBP in the control group. The average MBP in the Botulinum neurotoxin serotype A in gel phosphate buffer 9600 dose levels rose slightly above baseline level in the first hour immediately post-dosing, dropped significantly in the 2\textsuperscript{nd} hour post-dosing, and then rose back to the MBP level in line with other dose levels. The average MBP for the other Botulinum neurotoxin serotype A in gel phosphate buffer dose groups dropped significantly and continuously in hours 1 and 2 immediately post-dosing, and then rose and then dropped back to the level of first hour immediately post-dosing and remained relatively steady after 4 hours post-dosing but still below
the original baseline level. Except for the Botulinum neurotoxin serotype A in gel phosphate buffer 230 dose group, the control group had lowest average MBP after 8 hours post-dosing. Among all Botulinum neurotoxin serotype A in gel phosphate buffer dose groups, the Botulinum neurotoxin serotype A in gel phosphate buffer 230 dose group had the lowest average DBP between 4 and 16 hours post-dosing. It should be noted that the significant drop in MBP near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.

**Pulse Pressure (PBP)**

There was a significant dose effect on PBP at 20, 21, and 22 hours post-dosing for the for Botulinum neurotoxin serotype A in 2% milk groups. The average PBP at the 9600 and 31000 dose levels in the for Botulinum neurotoxin serotype A in 2% milk groups were significantly different from the control group at 20 and 21 hours post-dosing. The average PBP at the for Botulinum neurotoxin serotype A in 2% milk 9600 dose level was significantly different from the control at 22 hours post-dosing. The average PBP dropped significantly in the first two hours post-dosing for the for Botulinum neurotoxin serotype A in 2% milk group before gradually rising back to the baseline level after 8 hours post-dosing. One exception is the for Botulinum neurotoxin serotype A in 2% milk 180 dose group whose PBP rose over the baseline level at 6 hours post-dosing and stayed above baseline level until 16 hours post-dosing. From 10 to 24 hours post-dosing, the average PBP was consistently higher than baseline for for Botulinum neurotoxin serotype A in 2% milk dose groups 330, 3000, and 9600. Generally, the control group had a lower average PBP after 8 hours post-dosing in comparison to the for Botulinum neurotoxin serotype A in 2% milk group.
For the Botulinum neurotoxin serotype A in gel phosphate buffer, a significant dose effect on PBP was shown from 14 to 22 hours post-dosing. Pairwise comparisons show that the PBP in the 9600 and/or 31000 Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were significantly different from PBP in the control group. The average PBP dropped significantly in the first two hours post-dosing for the Botulinum neurotoxin serotype A in gel phosphate buffer group, and for most dose groups, gradually rose back to the baseline level and remained steady after 8 hours post-dosing. The exceptions were in the Botulinum neurotoxin serotype A in gel phosphate buffer 50 dose group whose average PBP level remained well below baseline level after received the toxin and the three highest Botulinum neurotoxin serotype A in gel phosphate buffer dose groups, 3000, 9600, and 31000 whose average PBP level rose past the baseline level after 11 hours post-dosing and continued rising for at least 5 hours. It should be noted that the significant drop in PBP near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.

**Heart Rate**

There was a significant dose effect on heart rate at 2, 12, 13, and 15 to 24 hours post-dosing for the Botulinum neurotoxin serotype A in 2% milk groups. However, at the 2 and 15 hours post-dosing, pairwise comparisons show these heart rates in the various Botulinum neurotoxin serotype A in 2% milk dose levels were not significantly different from the heart rate in the control group. The average heart rate at the 31000 dose level in Botulinum neurotoxin serotype A in 2% milk was significantly different from the control group at 12 and 13 hours post-dosing. The average heart rate at the 9600 dose level in Botulinum neurotoxin serotype A in 2% milk was significantly different from the control group at 16 to 24 hours post-dosing. The
average heart rate at the 3000 dose level in Botulinum neurotoxin serotype A in 2% milk was significantly different from the control at 21 to 24 hours post-dosing. The average heart rate dropped significantly in the first hour immediately post-dosing for the Botulinum neurotoxin serotype A in 2% milk group, rose back to baseline level between 2–3 hours post-dosing, and continued to rise above the baseline level after 3 hours post-dosing. In the low dose groups (50, 160, 180), the heart rate gradually decreased back to baseline levels after 20 hours post-dosing. In the high dose groups (3000, 9600, 31000), the heart rates were lower than the control group between 4-6 hours post-dosing. At about 7 hours post-dosing, the heart rate in animals in the highest dose group (31000) raised past the levels in the control group and gradually dropped until animal died. At about 11 hours post-dosing, heart rates in animals in the second highest dose group (9600) raised past the levels in the control group and gradually dropped until animal died. At about 12 hours post-dosing, heart rates in animals in the third highest dose group (3000) raised past levels in the control group and remained strong for at least 8 hours until gradually dropping prior to death.

There was a significant dose effect on the heart rate at 13 to 24 hours post-dosing for the Botulinum neurotoxin serotype A in gel phosphate buffer. However, at 13 hours post-dosing, pairwise comparisons show that the heart rates in the various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were not significantly different from the heart rate in the control group. The average heart rate at the 31000 dose level in Botulinum neurotoxin serotype A in gel phosphate buffer was significantly different from the control group at 14 to 18 hours post-dosing. The average heart rate at the dose level of 9600 in Botulinum neurotoxin serotype A in gel phosphate buffer was significantly different from the control group at 15 to 24 hours post-dosing. The average heart rate at the dose level of 3000 in Botulinum neurotoxin serotype
A in gel phosphate buffer was significantly different from the control group at 19 to 24 hours post-dosing. The average heart rate dropped significantly in the first hour immediately post-dosing for the Botulinum neurotoxin serotype A in gel phosphate buffer group, rose back to baseline level between 2–3 hours post-dosing, and then continued to rise above baseline level after 4 hours post-dosing. For the low dose groups (50, 160, 180), similar to the Botulinum neurotoxin serotype A in 2% milk groups, the heart rate gradually decreased back close to baseline level after 20 hours post-dosing. For two highest dose groups (9600 and 31000), the heart rates were lower than the control group from 3 hours post-dosing until death. It should be noted that the significant drop in heart rate near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.

Figure 15. Graph is illustrating the heart rate for an animal dosed in the Milk Dose Level 9.4x10² group.
Respiratory Rate

There was a significant dose effect on respiratory rate at 2, 3, 11, 12, 14 to 21, and 24 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk. However, at the 2, 3, 14, 15, 19, 20, 21, and 24 hours post-dosing, pairwise comparisons showed that the respiratory rates in various Botulinum neurotoxin serotype A in 2% milk dose levels were not significantly different from the respiratory rate in the control group. The average respiratory rate at the 31000 dose level in Botulinum neurotoxin serotype A in 2% milk was significantly different from the control at 11 and 12 hours post-dosing. The average respiratory rate at the 9600 dose level in Botulinum neurotoxin serotype A in 2% milk was significantly different from the control group at 16 to 18 hours post-dosing. For the control group, the average respiratory rate dropped significantly in the first hour immediately post-dosing, rose back close to the baseline level at about 3 hours post-dosing, and then remained stable throughout the 24 hours. For the lowest dose group (50), the average respiratory rate was both up and down after 2 hours post-dosing.
For the other two low dose groups (160 and 180), the average respiratory rate dropped slightly within the first hour post-dosing rose above the baseline level between 2-3 hours post-dosing, and generally remained stable after that. For one medium dose group (520), the average respiratory rate dropped slightly within the first hour post-dosing, rose above the baseline level at about 3 hours post-dosing, and generally remained stable after that. For the other three medium dose groups (290, 330, and 940), the average respiratory rate showed little change immediately after dosing, rose above baseline after 2-3 hours post-dosing and generally remained stable after that. At about 7 hours post-dosing, animals in the highest dose group (31000), their average respiratory rate started to drop until animal died. At about 8 hours post-dosing, the average respiratory rate in animals in the second highest dose group (9600) dropped until the animal died.

There was a significant dose effect on respiratory rate at 3 to 6 and 9 to 23 hours post-dosing for Botulinum neurotoxin serotype A in gel phosphate buffer. However, at 3 to 6, 9, 11, 16, 19, 20, 21, and 23 hours post-dosing, pairwise comparisons did not show significant differences of the various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels from the respiratory rate in the control group. The average respiratory rate at the 31000 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control at 10, 12, 13 and 14 hours post-dosing. The average respiratory rate at the 9600 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control group at 15 to 18 hours post-dosing. The average respiratory rate at the 3000 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control at 17 to 22 hours post-dosing. For the control group, the average respiratory rate dropped significantly in the first hour immediately post-dosing, rose back near the baseline level at about 3 hours post-dosing, and then remained relatively stable throughout 24 hours. For
the two lowest dose groups (50 and 160), the average respiratory rate was up and down from 4 hours post-dosing until the 24 hour time point. In the other low dose group (230), the average respiratory rate dropped slightly during the first hour post-dosing, rose immediately above baseline level, and then remained higher than baseline. For one medium dose group (520), the average respiratory rate dropped slightly within the first hour post-dosing and remained below baseline throughout 24 hours. For the other two medium dose groups (290 and 360), the average respiratory rate dropped slightly after dosing but rose above baseline after 2-3 hours post-dosing and then remained stable. Beginning at about 7, 9, or 10 hours post-dosing, the average respiratory rate dropped until death in animals in the dose group 31000, 9600, and 3000, respectively. It should be noted that the significant drop in respiratory rate near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.

**QA Interval**

There was a significant dose effect on QA interval at 2 to 10, 18, 20, and 23 hours post-dosing for Botulinum neurotoxin serotype A in 2% milk. However, except at 2 hours post-dosing, pairwise comparisons showed that the QA intervals in various Botulinum neurotoxin serotype A in 2% milk dose levels were not significantly different from QA intervals in the control group. At 2 hours post-dosing, the average QA interval at the 940 Botulinum neurotoxin serotype A in 2% milk dose level was significantly different from the control group. The average QA interval in the Botulinum neurotoxin serotype A in 2% milk dose groups increased significantly in the first hour immediately post-dosing, dropped significantly for the next 2 hours, continued to decrease below baseline until 7 hours post-dosing, and remained steady thereafter.
There was a significant dose effect on QA interval at 1, 3, 4, 8 to 10, and 13 to 21 hours post-dosing for Botulinum neurotoxin serotype A in gel phosphate buffer. However, except for 1, 8, and 9 hours post-dosing, pairwise comparisons showed that the QA intervals in the various Botulinum neurotoxin serotype A in gel phosphate buffer dose levels were not significantly different from QA intervals in the control group. At 1 hour post-dosing, the average QA interval at the 9600 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control. At 8 and 9 hours post-dosing, the average QA interval at the 230 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control. The average QA interval in the Botulinum neurotoxin serotype A in gel phosphate buffer dose groups increased significantly in the first hour immediately post-dosing, dropped significantly for the next 2 hours, continued to decrease below baseline at about 7 hours post-dosing, and for most Botulinum neurotoxin serotype A in gel phosphate buffer dose groups, remained steady below baseline afterwards. The average QA interval levels in the Botulinum neurotoxin serotype A in gel phosphate buffer 50 dose group rose above baseline level at about 17 hours post-dosing and stayed above baseline throughout 24 hours. The average QA interval levels in the Botulinum neurotoxin serotype A in gel phosphate buffer 520 dose group rose slightly above baseline level at about 8 hours post-dosing, dropped back to baseline in the next three hours, rose and stayed above baseline for the remainder of the 24 hours. It should be noted that the significant increase in QA interval near the time of dosing was likely due to the effects caused by the administration of the anesthetic agents.
Activity

One significant dose effect for Botulinum neurotoxin serotype A in 2% milk on activity level was found (8 hours post-dosing). However, pairwise comparisons showed the activity level at 8 hours post-dosing in various Botulinum neurotoxin serotype A in 2% milk dose levels were not significantly different from the activity level in the control group. The average activity level in the Botulinum neurotoxin serotype A in 2% milk dose groups decreased slightly in the first 7 hours post-dosing and rose above baseline at 8 hours for all dose levels prior to dropping back close to baseline levels the next hour.

Only one significant dose effect for Botulinum neurotoxin serotype A in gel phosphate buffer on activity level was found (8 hours post-dosing). Pairwise comparisons showed that at 8 hours post-dosing, the average activity level at the 3000 Botulinum neurotoxin serotype A in gel phosphate buffer dose level was significantly different from the control group. The average activity level jumped 8 hours post-dosing only in the Botulinum neurotoxin serotype A in gel phosphate buffer 160 and 230 dose groups. Other dose groups did not show a jump in activity level and remained below the baseline levels throughout the 24 hours post-dosing. The lowest activity level was found in the Botulinum neurotoxin serotype A in gel phosphate buffer 3000 dose group.

ECG

ECG findings showed clear and obvious changes in rates and rhythms attributable to the Botulinum neurotoxin serotype A; however, there appeared to be no differences in ECG’s between formulations of Botulinum neurotoxin serotype A in gel phosphate buffer or in 2% milk. ECG’s showed a slight increase in heart rate approximately 24 hours prior to death. At approximately 6 to 8 hours before death changes in ECG’s showed: 1) a decrease in heart rate
which became slower with time, 2) as the heart rate slowed, the sinus arrhythmia became more exaggerated, 3) J point depression and or change in configuration of ST-T generally became greater with time, 4) supraventricular and ventricular premature depolarizations, and short periods of paroxysmal ventricular tachycardia with one incidence of ventricular bigeminy, and 5) rare incidences of 2\textsuperscript{nd} degree AV block. Approximately 1 hour before death heart rate slowed precipitously, and ST-T became more bizarre where the J point was dramatically depressed.

At death, guinea pig number 57 developed either torsades de pointes or ventricular fibrillation, but then developed sinus arrest or 3\textsuperscript{rd} degree AV block with a slow idio-ventricular rhythm. All guinea pigs developed the sinus arrest except for animal number 144. Guinea pig number 144 had no substantive changes in ECG’s, and at the time of death the ECG appeared to be within normal limits.
Figure 17. Baseline Reading for an animal in the dose level 5.2x10^2 group.

Figure 18. 6 Hours Prior to Death for dose level 5.2x10^2, shows a decrease in heart rate with sinus arrhythmia.
Necropsy and Histopathology

Complete gross necropsies were performed on all animals (except #20 and #119) during the seven day observation period when found dead or euthanized. All surviving animals at the end of the seven day period were also necropsied. As expected no animals died due to disease and only incidental gross findings were observed. In phase 1 animal #121 had dark red diffuse discoloration of the lungs, #100 had dark red diffuse discoloration of the lungs and a cyst on the left uterine horn, #8 was found to show dilatation in the right kidney of approximately five times the normal size, and #101 and #9 had approximately 20 milliliters of fluid in the abdominal cavity. In phase 2, animal #131 and #44 had approximately 20 – 30 milliliters of fluid in the abdominal cavity. No gross findings were observed in animals from phase 3.
DISCUSSION

In this study, we assessed the protective qualities provided to Botulinum neurotoxin serotype A by fresh 2% milk against degradation in the gastrointestinal tract (e.g. stomach) of male and female Hartley guinea pigs. This assessment was used to see if the milk would aid in lowering the oral LD50 values in comparison to Botulinum neurotoxin serotype A in gel phosphate buffer (the traditional dosing solution). We also viewed the clinical observations and the pathophysiologic effects through the utilization of telemetry. Each animal that transmitted a signal was monitored for post-dosing alterations in mean, systolic, diastolic, and pulse pressures, ECG, temperature, activity, respiratory rate, QA interval, and heart rate.

Clinical observations taken for 7 days post-dosing showed few to no signs for animals in the three highest dose groups $3.0 \times 10^3$, $9.6 \times 10^3$, and $3.1 \times 10^4$ for animals dosed with Botulinum neurotoxin serotype A in milk or gel phosphate buffer. The animals from the gel phosphate buffer groups died less than 24 hours following dosing and were found dead the following morning. Animals in the two higher milk groups were found dead within 24 hours, however the dose group $3.0 \times 10^3$ had animals showing signs at 24 hours and were found dead within 48 hours. Animals that died between 24 and 48 hours most commonly showed signs of dyspnea, lethargy, paresis, and dehydration (a result of rapid onset of the disease and inability for the animals to reach water due to paralysis). Groups from all three phases that received the higher doses and had deaths occurring beyond 48 hours of dosing showed signs over several observation periods and/or days with the most common being dyspnea, lethargy, and rough hair coat. There were a few animals, mostly in the low dose groups, that showed signs and did not succumb to the toxins effects. These animals were in groups 1 and 6 from phase 1, group 16 from phase 2 and groups 20, 21, and 22 from phase 3.
A primary objective of this study was to assess the mortality rate, time to death, and LD₅₀ values for Botulinum toxin dosed in fresh 2% milk or gel phosphate buffer. Mortality rates were shown to be dose-dependent among all phases except group 13 in phase 2 where the lethality rate was higher than observed in the previous three higher doses. The average time to death could be predicted by the dose level, but was not significantly different between the two treatment groups. The milk appeared to have no protective factor against Botulinum neurotoxin serotype A.

The LD₅₀ ratio between the two treatment groups was computed in order to assess the toxicity of Botulinum neurotoxin serotype A in 2% milk in comparison to gel phosphate buffer. The LD₅₀ for Botulinum neurotoxin serotype A in 2% milk was 322.8 with a confidence interval of (193.4, 571.8) and for Botulinum neurotoxin serotype A in gel phosphate buffer the LD₅₀ was 349.0 with a confidence interval of (275.2, 479.6). Although the confidence intervals overlap, the point estimates at the LD₉₀ and LD₉₅ are 2 to 3 fold greater for the 2% milk buffer and the confidence intervals are wider. The test for the LD₅₀ ratio indicated that the ratio did not significantly differ from one another (p-value=0.7502) which indicated that the LD₅₀s do not differ. Although the LD₅₀ was lower in the milk groups there was no indication that the milk was protective for the Botulinum neurotoxin serotype A and was not statistically significant. The protection for the toxin could have been from the amount of fat in the milk that increased the transit time through the stomach and rate of absorption.

Pre- and post-dosing data collected on this study by the telemetry system included mean, systolic, diastolic, and pulse pressure, ECG, QA interval, temperature, activity, respiratory rate, and heart rate.

Body temperature has shown to be a highly predictable indicator of death. For the high dose levels the average body temperatures dropped significantly after ~10 to 12 hours and
continued to drop until death. In the high dose levels, when the body temperature began to drop, it appeared to be more rapid in the Botulinum neurotoxin serotype A in gel phosphate buffer than in Botulinum neurotoxin serotype A in 2% milk. For the mean of the animals in the high dose group of Botulinum neurotoxin serotype A in gel phosphate buffer, when the temperature dropped it never came back up as compared to the Botulinum neurotoxin serotype A in milk where the temperatures fluctuated up and down.

Blood pressure measurement effects were also dependent on the dose levels of toxin, but not the formulation in which the toxin was delivered. In general, when comparing dose levels, the high dose levels had higher average pressures than medium dose levels and low dose levels. All pressures dropped immediately post dosing most likely due to the affects of the anesthetic used prior to oral gavage. By 8 hours post-dosing, many Botulinum neurotoxin serotype A in milk and Botulinum neurotoxin serotype A in gel phosphate buffer dose levels had lower average SBP than the control group.

Heart rates and respiratory rates were also found to be good indicators of death. The three highest dose levels showed a dose dependent effect for Botulinum neurotoxin serotype A in both gel phosphate buffer and milk. As the animals recovered from anesthesia post dosing, their heart rates and respiratory rates increased above baseline levels. When death was imminent they gradually decreased and continued dropping until death. Both the increase and decrease to death are indicative of disease caused by the toxin.

The telemetry data showed that if the observations would have gone longer, more animals could have succumbed to death; although animals were observed as normal their telemetry data showed decreasing temperature, blood pressures and slowing heart rates.
The results of the ECG's showed that the toxin had an affect on the functions of the heart, but the extreme bradycardia most likely reflected terminal hypoxia resulting from respiratory arrest. The respiratory arrest would have resulted from the dosing of the Botulinum neurotoxin serotype A in gel phosphate buffer or in milk.

Increases in the heart rate likely resulted from one or a combination of factors including pain, fever, excitement, a drug effect on baroreceptor function; decrease in systemic arterial pressure, or by a direct effect on ion channels specific for SA nodal automaticity. The reduction in heart rate could have been caused by the opposite effects or to hypothermia. This would be relative to the progression of disease post-dosing. The augmentations of sinus arrhythmia and apparent gasping (indicated on the ECG’s by the voltage fluctuations and muscle tremor artifact) were likely interrelated.

The retained stability of the toxin in milk indicates that the introduction of the toxin into a supply of milk could cause significant morbidity, mortality, and impact on the economy due to the resulting panic. The importance of this study doesn’t only come from the results of the clinical signs, but from the amount of telemetry data collected on the guinea pig model. Between the clinical observations and the use of telemetry implants, Botulinum toxin serotype A was shown to be the cause of death. The telemetry data also gives baseline data for guinea pigs prior to dosing that can be referenced in other guinea pig studies.

From this study we cannot have a full understanding of the properties of milk that will directly affect the toxicity level of the Botulinum toxin serotype A. Based on the findings of mortality rates, time to death and onset of disease this study indicates that there is no significant change in the toxicity of Botulinum toxin serotype A whether delivered in fresh pasteurized 2% milk or in gel phosphate buffer. Mortality rates are dose dependent no matter which treatment
the guinea pigs received and time to death can be predicted by dose level. These results conclude that no change was evident to the Botulinum toxin serotype A over a 24 hour period.
Reference List


