Abstract

Introduction
The treatment of oesophageal atresia is challenging. The main goal is to achieve primary anastomosis. We have previously demonstrated in a pig model that intramural injection of botulinum toxin type A (BTX-A) resulted in significant elongation of the oesophagus during tensioning until bursting point. The objectives of the present study were to investigate the influence of different amounts of intramural BTX-A on the stretch-tension characteristics and histological changes of the oesophagus in piglets.

Materials and Methods
Fifty-two piglets were randomised to four groups receiving 2, 4 or 8 units/kg of BTX-A or isotonic saline (placebo). After 1 hour of rest the oesophagus was harvested and subjected to a stretch-tension test and histological examination to assess changes in the density of presynaptic vesicles in the nerve cells.

Results
Nine of the 52 animals were excluded from analysis due to problems with the stretch-tension test or death from anaesthesia. The maximum loads were higher in the BTX-A groups (2 units/kg: +2.1 N; 4 units/kg: +1.3 N; 8 units/kg: +1.9 N) than the placebo (p=0.046). There were no significant differences in percentage elongation, or histology.

Conclusions
This study demonstrated that injection of 2 units/kg BTX-A into a non-anastomosed oesophageal wall resulted in a modest increase in the maximum load achieved before bursting; this may be due to the muscle-relaxant effect of BTX-A. BTX-A injection produced no significant effects on elongation or oesophageal histology. The clinical usefulness of BTX-A in treatment of OA is still unclear.
Introduction

The treatment of oesophageal atresia (OA) is challenging and may have short- and long-term consequences for the patient, especially if there are postoperative complications. The main goal is to achieve primary anastomosis. Approximately 15% of children with OA have long gap OA (LGOA) making it difficult or even impossible to achieve primary anastomosis; in these cases significant tension is required and there is an increased risk of anastomotic leakage. Reduced anastomotic tension has been reported after circular myotomy in animal studies but results in human’s studies have been inconsistent.

Several different surgical methods have been used to treat cases of LGOA where primary anastomosis is not feasible, most commonly primary gastric transposition, colon interposition and secondary anastomosis following spontaneous growth of the upper and lower pouch and use of elongation techniques but all these procedures are associated with serious side effects. Gastric transposition leads to significant swallowing problems and gastric dysfunction in one third of patients. Colonic interposition may result in long-term functional problems and is associated with a high frequency of early postoperative complications. Secondary anastomosis requires a long hospital stay during which the patient undergoes continuous suction on the upper pouch and placement of a gastrostomy tube for enteral feeding. Finally, these patients have a higher incidence of gastro-oesophageal reflux disease in later life.

We previously demonstrated in an animal study that in comparison with placebo an intramural injection of botulinum toxin type A (BTX-A) resulted a significant elongation of the oesophagus during tensioning until bursting point. In addition two case reports with LGOA, including one from our institution, demonstrated that injection of BTX-A into the oesophageal wall of the pouches produced sufficient relaxation to enable anastomosis to be achieved. The administered dose was on an empiric bases, and there is a need for further studies on dose-response, not only from a clinical point of view but also from at toxicological. In addition it is important to know the effect or short oesophageal segments. The present study showed the result of the cylindrical stretch, which may be different when compared to patch stretch. The method used in the present study was chosen to resemble the clinical situation.

BTX-A is derived from the gram-positive bacteria Clostridium botulinum. It is well known that BTX-A blocks acetylcholine release at the neuromuscular junctions thus preventing the vesicle containing acetylcholine from fusing with the presynaptic membrane and causing muscle relaxation. The timeframe of this effect is unknown.

The objectives of this study were therefore 1) to establish the dose-response curve for use of intramural injection of BTX-A to achieve elongation of oesophagus during tensioning to bursting point and 2) to examine BTX-A-induced changes in the density of vesicles in nerve terminals in piglets.

Material and Methods

Design

The animals were randomised to a control group (saline group) and 3 different treatment groups, which received intramural injections of BTX-A at various concentrations. The total volume of
injected fluid (2 ml) was the same in all experimental groups. After a 1-hour rest the oesophagus was harvested and subjected to a stretch-tension test and histological assessment.

2.2 Animals
Three-week-old male suckling pigs were used. The pigs were castrated when they were 3 days old. The animals were housed under heating lamps from the time they arrived at the facility until the operation. No food was provided for four hours prior to surgery to avoid aspiration.

2.3 Anaesthetic and analgesic protocol
The pigs were sedated using a mixture of midazolam at 0.25 mg/kg (Dormicum®, 5 mg/ml, Hameln), medetomidin at 0.05 mg/kg (Sedator®, 1 mg/ml, Novartis), and atropine at 0.05 mg/kg (1mg/ml, Amgros I/S) administered intramuscularly. Induction of anaesthesia was performed using a mixture of midazolam at 1.5 mg/kg and ketamine at 7.5 mg/kg (Ketaminol® Vet, 100mg/ml, MSD Animal Health) administered intravenously using a 22-gauge Venflon® inserted in an ear vein. The pigs were intubated with a cuffed tube, size 4.0, and anaesthesia was maintained using 2.2 isoflurane administered via an MCM 801 ventilator (Dameca, Rødovre, Denmark) using a 2:1 mixture of oxygen and air. The animals were ventilated at 16 breaths per minute with a tidal volume of 10 ml/kg. Blood pressure, electrocardiographic parameters, heart rate and oxygen saturation were monitored continuously (NONIN 8500V, Nonin Medical, Minnesota, USA or Dräger Infinity Gamma XL, Dreager Medical Canada Inc.). Buprenorphine at 0.015 mg/kg (Temgesic®, 0.3 mg/ml, Reckitt Benckiser) was given intravenously prior to skin incision for analgesia. The pigs were placed on a heating pad set to 32 °C (Gaymar T/Pump, Gaymar Industries, Inc. NY, USA) to avoid hypothermia during surgery. At the end of study the pigs were euthanised with an intracardial injection of pentobarbital (Pentobarbital, 200mg/ml, Glostrup Apotek, Denmark).

2.4 Surgical procedure
The oesophagus was exposed through a right-sided thoracotomy. A 5-cm-long segment in mid-oesophagus was delineated proximally and distally with clips (Endopath® EMS, Ethicon endosurgery, Inc., Cincinnati, USA). A solution containing isotonic saline or 2, 4 or 8 units/kg BTX-A (Xeomin®, Merz Pharmaceuticals GmbH, Frankfurt/Main, Germany) was injected intramuscularly in depots of 0.1 ml. Four injections were administered in a quadrant pattern at 15, 20, 25, 30 and 35 mm from the proximal clips. The duration of cold ischaemia (period between removal of oesophagus and the start of stretch-tension analysis) was noted.

2.5 Stretch–tension test
After one hour of observation the oesophagus was removed en bloc and the 5-cm-long marked segment was mounted in a stretch-tension device (LFPlus Series Universal Test Machine, Lloyd Instruments LTD, Hampshire, UK). The specimen was subjected to a 2 N preload to compensate for length difference in between the specimens. The oesophagus was then stretched at a constant deformation rate of 30 mm/min. The maximum load and elongation at preload and maximum load were derived from the load-strain curve by the software (Fig 1).

2.6 Histopathological analysis
After the stretch-tension test the oesophageal segment was fixed in 4% formalin. The samples were dehydrated and embedded in paraffin blocks; 4 levels from each specimen were used. Three-µm sections were cut and stained on an Autostainer (Ventana) for synaptophysin (Novocastra 27G12) 1:50 and S100 (Dako Z0311) 1:4000 and Haematoxylin-Eosin. The slides were examined to assess structural changes and the density of presynaptic vesicle in nerve cells. The pathologist was blind to
the treatment group to which samples belonged.

2.7 **Sample size estimation**
In previous studies an 18 % difference between treatment and placebo group has been observed. Assuming a standard deviation of 17 %, a significance level of $p = 0.05$ and a power of 80 %, we calculated that a group size of 12 animals would be needed to confirm this difference. We anticipated a maximum attrition rate of 10 % and therefore the total number of animals included was 52.

2.8 **Statistical analysis**
A multiple linear regression with maximum load as a function of BTX-A dose, weight, intraoperative saturation, mean intraoperative blood pressure, heart rate, elongation and cold ischaemia time was performed. P-values less than 0.05 were considered significant. Statistical calculations were performed using Stata Software (version 11, StataCorp LP, Texas, USA).

2.9 **Ethical considerations**
This study was approved by the Danish National Experimental Animal Board. Measures were taken to ensure that no animals suffered during the experimental procedure.

3 **Results**

3.1 **Baseline values**
Of the 52 animals included a total of 9 animals were excluded, 4 (1 in each group) died during anaesthesia and 5 (1 in the placebo group; 1 in the 2 units/kg group and 3 in the 8 units/kg group) owing to problems mounting the tissue specimen correctly in the stretch-tension device. Baseline values for the 43 animals included in the analysis are listed in Table 1. There were significant differences in weight and duration of cold ischaemia between the treatment groups and the placebo group. Otherwise there were no differences.

3.2 **Stretch-tension characteristics**
Elongation and percentage elongation against tension and maximum load are shown in Table 2. Load at bursting point was significant higher in the BTX-A groups (2 units/kg: +2.1 N; 4 units/kg: +1.3 N; 8 units/kg: +1.9 N) than the placebo ($p=0.046$). Table 2. Elongation and percentage elongation were similar in all groups Table 2.

3.3 **Histology**
The injection sites were identifiable by Haematoxylin-Eosin stained sections in all specimens. No changes in neural structures were observed in either the Haematoxylin-Eosin or S100 stained sections. Synaptophysin immunohistochemical staining did not reveal any significant changes in density of presynaptic vesicles in BTX injected groups in comparison with the placebo group.

4 **Discussion**
The mean difference in maximum load between the group of animals who received 2 units/kg and the placebo group was 2.1 N. This difference may be clinically relevant, especially in cases of heavy tension anastomosis. We hypothesise that BTX-A may reduce the risk of leakage and stricture formation in these cases, but clinical studies are needed to test this hypothesis. Another important finding was that an increase in the dose of BTX-A above 2 units/kg had no additional effect; this has implications for treatment safety in humans. We note that no serious adverse events defined as significant changes in vital parameters were observed in this study. The dose in the two published case histories was 4 units/kg\(^\text{18}\); no serious adverse events were observed in those cases, but the side-effect profile of BTX-A in babies is unknown. The most important side-effects will be neuromuscular or cardiopulmonary.

In a previous similar study conducted at our institution\(^\text{17}\) BTX-A injection was associated with a difference in oesophageal elongation whereas in this study BTX-A did not appear to affect elongation. A difference in the experimental procedure may account for these divergent results. In the previous study the entire oesophagus was harvested in a way that allowed the entire 5 cm segment of prepared oesophagus to be stretched, whereas in this study the prepared segment was only 5 cm in length, leaving only about 3 cm for testing. This procedure was used to test whether it is possible to obtain similar results on shorter oesophageal segments. Taken together the results of these two studies demonstrate that intramural injection of BTX-A has positive effects on stretch characteristics of the oesophageal pouches, but the effect is more evident in longer pouches, meaning that the technique may be doubtful in cases where the distal pouch is very short.

Histological assessments in this study showed that in piglet intramural injection can be used successfully; although the oesophageal wall is thicker in piglets than babies there are no indications that the success rate would be dissimilar in babies. Another important finding was that the injections only caused a slight local oedema and no detectable tissue damage. The known effects of BTX-A\(^\text{19,20}\) suggest that BTX-A treatment might lead to an accumulation of synaptic vesicles, but we did not observe any increase in density of the vesicle protein, synaptophysin. This may have been due to the short observation period, only 1 hour. In other studies increases in vesicle numbers have been observed after 3 to 15 days.

4.1 Methodological considerations and limitations of the study
There were group differences in the duration of cold ischaemia and in weight although group assignment was random and blinded for the investigator. Table 1 shows that the difference in duration of cold ischaemia was only a few seconds and it is doubtful that this has affected the overall results. The higher weight of the piglets in the placebo group might in theory have influenced the results. The small, non-significant group differences in heart rate, saturation and blood pressure provide evidence for the safety of the method. Detached oesophagus is different from anastomosed oesophagus so direct parallels in to a clinical setting is not possible from this study.

The proportion of animals excluded from analysis was higher (17\%) than we had anticipated in calculating sample size. We chose to exclude all animals (5) in which the stretch-tension test did not succeed the first time because the results of a second attempt might be affected by damage to the tissue during the first attempt.

4.2 Future research
1) Studies on the effect of BTX-A in a chronic high-tension anastomotic animal model would be ideal to test the consequences of BTX-A over time.
2) Animal study on the time course of the effects of BTX-A injections on the strength-tension properties of oesophageal tissue should be performed to determine the ideal latency at which to attempt primary anastomosis before this technique is tested in humans.

5 Conclusion

This study demonstrated that injection of 2 units/kg BTX-A into a non-anastomosed oesophageal wall resulted in a modest increase in the maximum load achieved before bursting; this may be due to the muscle-relaxant effect of BTX-A. BTX-A injection produced no significant effects on elongation or oesophageal histology. The clinical usefulness of BTX-A in treatment of OA is still unclear.

6 Acknowledgments

Veterinarian Jakob le Fèvre Harslund and Head of Biomedical Laboratory Peter Bollen, The Biomedical Laboratory, University of Southern Denmark

7 References


Figure 1: Example of a load strain curve with instructions of elongation, preload and maxload
Table 1: Baseline values in the three treatment groups (BTX-A) and the placebo group. Values are mean ± standard deviation.

<table>
<thead>
<tr>
<th>BTX-A in units/kg</th>
<th>Placebo</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 12</td>
<td>n = 9</td>
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<tr>
<td>Weight</td>
<td>6.2 ±0.27</td>
<td>6.0 ±0.2</td>
<td>6.2 ±0.3</td>
<td>5.9 ±0.3</td>
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<tr>
<td>Oxygen saturation**</td>
<td>97.8 ±2.0</td>
<td>98.1 ±1.1</td>
<td>97.1 ±2.3</td>
<td>97.2 ±2.3</td>
<td>0.182</td>
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<td>Harte rate**</td>
<td>120.2 ±16.2</td>
<td>124.1 ±13.1</td>
<td>128.0 ±13.3</td>
<td>125.4 ±11.4</td>
<td>0.091</td>
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<td>Blood pressure**</td>
<td>60.2 ±12.2</td>
<td>64.5 ±10.6</td>
<td>59.3 ±9.7</td>
<td>56.8 ±6.4</td>
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<td>Cold ischemia</td>
<td>238.6 ±99.6</td>
<td>219.8 ±48.6</td>
<td>273.4 ±131.4</td>
<td>238.3 ±118.2</td>
<td>0.023</td>
</tr>
</tbody>
</table>

** Mean oxygen saturation, heart rate and mean blood pressure during the experimental procedure.
- n = number of animals
- Cold ischaemia = time in seconds from removal of the oesophagus to start of the stretch test.
- There was 1 missing value in data on heart rate and oxygen saturation
- There were 7 missing values in data on blood pressure
Table 2: Mean oesophageal elongation, % elongation and maximum load.

<table>
<thead>
<tr>
<th>BTX-A (units/kg)</th>
<th>Mean</th>
<th>Std deviation</th>
<th>Range</th>
<th>p-value</th>
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<tr>
<td>Elongation (mm)*</td>
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<td></td>
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<tr>
<td>0</td>
<td>15.2</td>
<td>±3.8</td>
<td>9.0 - 21.3</td>
<td>0.224</td>
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<tr>
<td>2</td>
<td>15.1</td>
<td>±3.4</td>
<td>11.2 - 21.7</td>
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<tr>
<td>4</td>
<td>15.9</td>
<td>±4.2</td>
<td>10.3 - 24.6</td>
<td></td>
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<tr>
<td>8</td>
<td>16.0</td>
<td>±3.0</td>
<td>11.2 - 19.4</td>
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<tr>
<td>Elongation (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.222</td>
</tr>
<tr>
<td>0</td>
<td>71.5</td>
<td>±3.9</td>
<td>64.9 - 79.0</td>
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<tr>
<td>2</td>
<td>70.4</td>
<td>±4.0</td>
<td>62.3 - 78.5</td>
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</tr>
<tr>
<td>4</td>
<td>71.4</td>
<td>±4.0</td>
<td>62.3 - 78.5</td>
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<tr>
<td>8</td>
<td>70.4</td>
<td>±3.0</td>
<td>64.7 - 74.4</td>
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<tr>
<td>Maximum load (N)</td>
<td></td>
<td></td>
<td></td>
<td>0.046</td>
</tr>
<tr>
<td>0</td>
<td>19.2</td>
<td>±5.1</td>
<td>11.4 - 26.1</td>
<td></td>
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<tr>
<td>2</td>
<td>21.3</td>
<td>±3.2</td>
<td>16.7 - 26.1</td>
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<tr>
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<td>20.5</td>
<td>±3.6</td>
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<tr>
<td>8</td>
<td>21.1</td>
<td>±3.1</td>
<td>15.9 - 25.2</td>
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</table>

* Elongation from preload to maximum load