Lower esophageal sphincter augmentation by endoscopic injection of dextranomer hyaluronic acid copolymer in a porcine gastroesophageal reflux disease model

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Background: We previously demonstrated feasibility, safety, and a reproducible histologic bulking effect after injection of dextranomer hyaluronic acid copolymer (DxHA) into the gastroesophageal junction of rabbits. In the current study, we investigated the potential for DxHA to augment the lower esophageal sphincter (LES) in a porcine model of gastroesophageal reflux disease (GERD).

Methods: Twelve Yucatan miniature pigs underwent LES manometry and 24-hour ambulatory pH monitoring at baseline, after cardiomyectomy, and 6 weeks after randomization to endoscopic injection of either DxHA or saline at the LES. After necropsy, the foregut, including injection sites, was histologically examined.

Results: Pigs in both groups had similar weight progression. Cardiomyectomy induced GERD in all animals, as measured by a rise in the median % of time pH < 5 from 0.6 to 11.6 (p = 0.02). Endoscopic injection of DxHA resulted in a higher median difference in LES length (1.8 cm vs. 0.4 cm, p = 0.03). In comparison with saline injection, DxHA resulted in a 120% increase in LES pressure, and 76% decrease in the mean duration of reflux episodes, but these results were not statistically significant. Injection of DxHA induced a foreign body reaction with fibroblasts and giant cells.

Conclusions: Porcine cardiomyectomy is a reproducible animal GERD model. Injection of DxHA may augment the LES, offering a potential therapeutic effect in GERD.

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induces a foreign body and fibrotic reaction at the site of injection. It does not migrate, is nonimmunogenic, and is stable for long periods with no reports of adverse long-term effects. Since its introduction into pediatric urologic practice in the late 1990s, this agent has resulted in a significant paradigm shift from surgical to endoscopic treatment of vesicoureteral reflux disease [22,23]. The agent has also been used for closure of recurrent tracheoesophageal fistula, umbilical hernia repair, and treatment of urinary and fecal incontinence [24–27].

Our group has been interested in a potential role for DxHA as a treatment for GERD. We have previously demonstrated feasibility, safety, and a predictable histologic bulking reaction after DxHA injection into the rabbit gastroesophageal junction (GEJ) [28]. The purpose of this study was to investigate the effect of the endoscopic injection of DxHA into the LES in a porcine GERD model.

1. Methods

1.1. Animal model

Fourteen Yucatan miniature swine weighing 8.4 ± 1.3 kg were used for the experiment. This strain was specifically chosen because of its slow growth curve. The swine were fed a basic research diet, Teklad Miniswine® (Harlan Laboratories, Indianapolis, IN). Pigs were started on Ensure® (Abbott Laboratories, North Chicago, IL) 48 hours before each procedure, and given water only for 24 hours prior to the procedure. All procedures were performed under general inhalational anesthesia with 2% isoflurane, following sedation with intramuscular injection of butorphanol (0.1 mg/kg), acepromazine (0.2 mg/kg), atropine (0.05 mg/kg), and ketamine (0.14 ml/kg).

Prior to initiating the study, manometry and 24-hour ambulatory pH monitoring were performed on two animals to confirm feasibility and ascertain that the tracings obtained can be interpreted by the research team. Subsequently, a laparoscopic cardiomyectomy was attempted on both pigs. However, both procedures required conversion to laparotomy in order to adequately identify the gastroesophageal junction and all the muscle layers. Subsequently, all the animals underwent open cardiomyectomy as described below. Fig. 1 depicts the experimental protocol.

Approval of the research protocol was obtained from the McGill University Animal Care Committee, Montreal General Hospital Site (2012–7056).

1.2. LES manometry

Esophageal manometry was performed using a commercial stationary water-perfused manometry system including a low-compliance pneumatic capillary infusion pump with a flow rate of 0.5 ml/min (model PIP-4-8, MUI Scientific, Mississauga, Ontario, Canada), a polygraph (Medtronic, Ontario, Canada), and a commercial computer. The four-channel water perfused esophageal catheter (PE4-3–3–3, MUI Scientific, Mississauga, Ontario, Canada) was used for manometry with four sensors 3 cm apart from each other and with a radial orientation of 90°. This allowed for the collection of four data sets for a given site within the esophagus with a single dynamic measurement. At each level along the esophagus, a mean pressure was obtained from all four sensors. Using a stationary pull-back technique, the catheter was manually pulled out at a speed of 5 mm/min through the LES, with registration of the pressure within the LES by all four sensors. The lower border of the LES was determined at the station that demonstrated a consistent rise of pressure above the gastric baseline pressure, whereas the upper border of the LES was determined at the station that showed a drop of pressure to esophageal baseline pressure. Before removing the catheter, the distance between the upper border of the LES and the snout was measured for the subsequent insertion of the pH probe. From the data obtained, which were automatically recorded as pressure curves via a polygraph on a standard PC using GastroTrac software (version 4.3.0.47, Alpine Biomed ApS, Skovlunde, Denmark), we measured the total length, abdominal length, and resting pressure of the LES, as previously described by Zaninotto et al. [29].

1.3. 24-hour ambulatory esophageal pH measurements

After completing the manometry and under the same general anesthetic, a single-sensor, internal reference pH catheter (MUI Scientific, Mississauga, Ontario, Canada) was used for 24-hour pH measurement. After successful calibration using a Digitrphr pH 400 (Medtronic, Minneapolis, MN), the probe was inserted through intravenous tubing to gain more rigidity, and avoid coiling in the nasopharynx. The tip of the probe was left exposed. Using multiple 2.0 silk sutures, the probe was fixed to the overtube to prevent the probe from sliding inside the tube, especially when the animal was ambulatory. Subsequently, the pH probe with overtube was inserted through the pig’s snout and guided into the esophagus with the aid of a laryngoscope. The tip of the probe was placed 2 cm above the manometrically identified upper border of the LES, and marked at the snout level to allow recognition of changes in its position. Fluoroscopy was used in the first few animals to document proper placement of the probe. The probe was then secured externally with several interrupted 0 silk sutures spaced 5 cm apart, starting from the snout and proceeding to the animal’s back, leaving enough slack to allow the animal freedom of movement without displacing the probe. Finally, a custom-made dorsal harness with a pocket was used to house the Digitraper. Upon recovery, the animal was allowed free access to water and food in a private cage to avoid disruption of the system by other pigs. After 24 hours, the sutures were cut and the probe was removed. Data were uploaded to a computer using GastroTrac software (version 4.3.0.47, Alpine Biomed ApS, Skovlunde, Denmark). Acid reflux was defined by a drop in esophageal pH below 5. Data obtained were number of reflux episodes, number of long refluxes (>5 minutes), longest reflux episode (minutes), total time pH < 5 (minutes), percentage of time pH < 5 (minutes), and mean duration of reflux episodes (minutes). DeMeester and Boix-Ochoa scores, modified to pH < 5, were calculated.

![Fig. 1. Experimental protocol. pH measurements all consisted of 24-hour ambulatory pH probe.](image-url)
1.4. Cardiomyectomy

Prophylactic cefazolin (20 mg/kg) was given intravenously. A14-French orogastric tube was inserted. The abdominal cavity was accessed through an upper midline laparotomy incision from the xiphoid to the umbilicus. The left lobe of liver and the spleen were retracted in order to expose the area of the GEJ. The phrenoesophageal membrane was incised, the anterior peritoneal reflection covering the esophagus was opened, and the anterior and posterior vagal trunks were identified and preserved. At the GEJ, an anterior longitudinal myotomy was performed by incising the longitudinal and the circular muscle fibers using a No. 15 blade and a fine Metzenbaum scissors. The myotomy was extended 3 cm cephalad on the esophagus and 3 cm caudad on the gastric cardia. Once the submucosal plane was entered, the esophageal muscle layers were gently peeled away 1 cm on both sides. Both pieces of muscle were then excised creating a myectomy segment that was 6 cm in length and 2 cm in width centered at the GEJ (Fig. 2). A very essential part of the myectomy was to ensure dividing the gastric sling and clasp muscle fibers, a step that required careful dissection and carried the highest risk of mucosal perforation. Once hemostasis was ensured, the abdomen was closed in the standard fashion with a monofilament suture. The skin was closed with subcuticular sutures to avoid the presence of any material externally. OpSite® waterproof spray was used as a dressing. The animal was awakened and extubated. Intramuscular buprenorphine 0.01–0.1 mg/kg was administered for analgesia. The pigs were allowed free access to food and water. Postoperatively, cephalexin (25 mg/kg) was administered orally twice a day for 10 days.

1.5. Endoscopic injections

A 2-week recovery period was allowed after surgery, during which computer-generated randomization to either DxHA or saline was performed. Under general anesthesia, a 45 cm custom-made rigid endoscope (Fiegert-Endotech, Sunrise, FL) was advanced through the pig’s mouth. After the GEJ was identified, a semirigid beveled needle was advanced through a working channel in the scope. Submucosal injections of DxHA or saline were performed in three different quadrants away from the muscle-deficient cardiomyectomy site to avoid injecting outside the esophagus. A volume of 1 ml of DxHA or saline was injected in each site. A 30-second waiting period was allowed for the implant to stabilize before withdrawing the needle. Immediate mucosal bulging indicated successful implantation (Fig. 3). Six animals had DxHA injections, whereas four animals received saline injections. After completing the injection session, the animal was awakened and extubated. Free access to water and food was allowed immediately after the procedure. During the ensuing

Fig. 2. Appearance of the completed cardiomyectomy. The mucosa is seen to bulge after the overlying muscle is removed.

Fig. 3. Endoscopic injection of DxHA at the GEJ. A volcano-like effect results owing to installation of the polymer in the submucosa and muscularis.

6 weeks, the animals were weighed weekly. Behavior and feeding patterns were recorded. At six weeks after injection, animals underwent a third manometry and 24-hour ambulatory pH measurement, prior to sacrifice and necropsy the following day.

1.6. Euthanasia and necropsy

After induction of anesthesia with 5% isoflurane, an intravenous injection of sodium pentobarbital (120 mg/kg) was used to euthanize the animal. Necropsy was performed through a midline sternotomy and laparotomy. En bloc resection of esophagus, stomach, liver, and lungs was performed.

1.7. Histological assessment

Gross and histological examinations were performed by two pathologists. Several sections were made from the GEJ at different levels and stained with hematoxylin ploxine saffron. Specimens were examined for esophagitis, response to the GEJ injection, type of cellular reaction, signs of implant migration, reaction outside the esophagus, stenosis, or perforation. Additionally, liver and lungs were examined for abnormal reaction and evidence of aspiration respectively.

1.8. Statistical analyses

Weight progression was compared using ANCOVA, controlling for the weight at time of injection. Paired continuous data were compared using paired Wilcoxon signed rank test. Unpaired comparison of continuous data was performed using Mann-Whitney U test. P-value of less than 0.05 was considered significant. Analysis was performed using IBM SPSS 20.0 and reviewed by a biostatistician. Pathology results were reported qualitatively.

2. Results

During the study period, two pigs randomized to saline injection died during the first week after uneventful cardiomyectomy, before endoscopic injection. One animal had recurrent vomiting, with aspiration pneumonia diagnosed on necropsy. The second animal had evidence of congestive heart failure of unclear etiology. The other 10 pigs continued to gain weight as shown in Fig. 4, with no significant difference between DxHA or saline groups as determined by ANCOVA (p = 0.98).

The manometry and pH measurements precardiomyectomy and postcardiomyectomy are shown in Table 1. Cardiomyectomy resulted
in reductions in LES pressure, total length, and abdominal length, but the differences did not reach statistical significance. Significantly increased acid exposure was demonstrated by increase in the total and percentage time pH less than 5.

When comparing the six pigs in the DxHA group and the 4 pigs in the saline group, there were no statistically significant differences in median LES pressure and percentage of time pH < 5 as determined by Mann-Whitney U test. In order to control for the difference between baseline values, another comparison was performed between postoperative and postinjection median differences. We noted a longer LES in the DxHA compared to saline group (1.8 cm vs. 0.4 cm, p = 0.03). This observation was consistent when performing subgroup analysis of the DxHA group only, comparing postoperative and post injection median LES length (1.8 cm vs. 3.3 cm, p = 0.06), as shown in Fig. 5.

We also calculated the percentage change in each median value between postmyectomy and postinjection data sets. In comparison to saline, DxHA had resulted in 120% increase in LES pressure (median change of pressure – 0.5 mm Hg in saline group, 0.1 mm Hg in DxHA group, p = 0.8), and 124% decrease in the number of reflux episodes (median change of number of reflux episodes – 22 in DxHA group, 91.5 in saline group, p = 0.6).

The histologic results are shown in Table 2. Gross examination of the specimens revealed an area of increased thickness with no luminal narrowing in DxHA injected animals. Microscopically, DxHA implants were identified tracking from the submucosa to the muscularis propria of all 6 specimens accompanied by foreign body fibrous tissue reaction with giant cells and collagen deposition (Fig. 6). No fibrous tissue reaction was seen in the saline group. In 4 out of 6 DxHA specimens, acute inflammatory cells (microabscess) were identified close to injection site. No evidence of perforation or periesophageal inflammation was noted. DxHA did not migrate above or below the site of injection. There was no histologic evidence of esophagitis in any of the specimens.

### Table 1

<table>
<thead>
<tr>
<th>Preoperative data</th>
<th>Postoperative data</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Manometry:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LES pressure (mm Hg)</td>
<td>1.35</td>
<td>1</td>
</tr>
<tr>
<td>LES total length (cm)</td>
<td>3.66</td>
<td>2.5</td>
</tr>
<tr>
<td>LES abdominal length (cm)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>PH measurement:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of refluxes</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Number of long refluxes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Time of pH &lt; 5 (min)</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Percentage of time pH &lt; 5</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Mean duration of reflux episodes (min)</td>
<td>0.29</td>
<td>0</td>
</tr>
<tr>
<td>Duration of longest reflux episode (min)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>DeMeester score</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Boix-Ochoa score</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Comparisons were performed using paired Wilcoxon signed rank test.
Nevertheless, there is still strong evidence that augmentation of LES pressure alone may be an effective antireflux measure. Ganz et al. recently reported three-year outcomes of a flexible magnetic ring that exerts its effect exclusively by augmenting LES pressure, showing effective treatment of GERD in most patients [32]. DxHA represents a potentially ideal injectable agent because of its appealing properties. Its main component, hyaluronic acid, is a naturally present compound that is nonimmunogenic. Studies have shown that DxHA does not migrate from the original site up to 3 years after injection [33]. A granulomatous inflammatory reaction and fibrotic encapsulation likely contribute to the durability of the implant long after DxHA is hydrolyzed by the body [23]. DxHA has been effectively used for more than two decades in the treatment of vesicoureteral reflux, and has been recently introduced for a variety of other pediatric applications [22,23,25–27]. In adults, transanal submucosal injection of DxHA has been shown to be effective for the management of fecal incontinence [24]. This application is particularly pertinent to our current work. Whereas DxHA injection in the bladder is thought to work by changing the vesicoureteral angle, injection in the anal sphincter is thought to be effective owing to higher sphincter pressure.

In previous work, we have found that injection of DxHA into the rabbit GEJ was well tolerated, and did not result in esophageal obstruction or perforation. Histologically, DxHA was able to induce fibrous tissue reaction at the GEJ, very similar to the reaction observed at the ureterovesical junction [28]. Eosinophilia observed in the rabbit esophagus after DxHA injection was not seen in the pig, and may be species specific. The rabbit foregut anatomy is remarkably similar to humans. However, despite several attempts, we were not able to create a reliable GERD model in the rabbit. The muscular layer of the GEJ in the rabbit is quite thin, rendering myectomy without perforation difficult. The rabbit esophagus is narrow, and endoscopic injection was not possible. In addition, the rabbit’s stomach never completely empties and always contains large bezoars from ingested hairs. Ambulatory pH measurements in rabbits were not possible.

Several GERD large animal models have been published, but few have been reproducible [34–41]. There is no consensus on the most reliable experimental procedure to induce GERD. However, a partial cardiomyectomy appears to be superior to cardiomyotomy with respect to the degree of acid reflux [34]. We developed our animal model based on pilot work in pigs reported by Schopf et al. [39]. We learned important lessons during development of our model. The porcine GEJ is quite complex because of well-developed clasp and sling oblique gastric fibers that act as a natural partial fundoplication. These fibers have to be divided to induce GERD. We found the dissection of the GEJ by laparoscopy to be quite difficult owing to the ease of entering the pleura and causing a pneumothorax. In addition, an adequate myectomy required significant mobilization of the GEJ and deliberate division of each muscular layer, which we found subsequently replaced by fibroblasts and collagen. A significant decrease in both GERD symptoms and esophageal acid exposure was achieved [19,31]. However, this agent was also withdrawn. To our knowledge, there is currently no injectable agent approved for the treatment of GERD.

Fig. 5. Box plots of the LES total length in the (A) DxHA group and (B) Saline group at baseline, post cardiomyectomy, and 6 weeks post injection. Transverse lines represent median values.

### Table 2
Histology results.

<table>
<thead>
<tr>
<th>Pigs</th>
<th>Injection type</th>
<th>DxHA implant present?</th>
<th>Foreign body reaction present?</th>
<th>Giant cells present?</th>
<th>Fibroblasts present?</th>
<th>Microabscesses present?</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No inflammation noted</td>
</tr>
<tr>
<td>2</td>
<td>Saline</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No inflammation noted</td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No inflammation noted</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No inflammation noted</td>
</tr>
<tr>
<td>5</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>DxHA identified outside the muscularis propria</td>
</tr>
<tr>
<td>6</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>DxHA identified in the muscularis propria. Presence of abscess with a small amount of DxHA outside the muscularis propria</td>
</tr>
<tr>
<td>7</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>DxHA tracks outside the muscularis propria</td>
</tr>
<tr>
<td>8</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>DxHA identified outside the muscularis propria Abscess identified in the submucosa</td>
</tr>
<tr>
<td>9</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>DxHA identified in the muscularis propria</td>
</tr>
<tr>
<td>10</td>
<td>DxHA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>DxHA present within a 4 cm abscess located outside the muscularis propria near the myotomy site</td>
</tr>
</tbody>
</table>
significantly more involved than in human operations. Nevertheless, others have reported achieving the same goal in pigs by laparoscopy [36]. Interestingly, despite a generous myectomy, we found identification of the myectomy site quite difficult on subsequent endoscopy. This may have prevented us from injecting exactly at the myectomy site, and may have influenced our results. Although quite involved, our procedure of 24-hour ambulatory pH monitoring in pigs was successful and reproducible. The lack of esophagitis seen in all animals did not allow for evaluation of this endpoint or its prevention. The survival period of six weeks after cardiomyectomy was likely too short for esophagitis to be demonstrated.

This study demonstrates the feasibility and safety of DxHA injection into the GEJ in a porcine GERT model. Implantation of DxHA at the GEJ is technically feasible using a rigid scope with a beveled needle. However, the tip of the needle must not traverse the esophageal wall. This occurred in some of our injections, with DxHA found outside the wall of the esophagus, in some cases surrounded by an inflammatory reaction. However, none of those pigs experienced any complications. Nevertheless, the endoscopic injection technique requires significant refinement. More accurate injection may be facilitated by use of a needle with limited extension, such as a sclerotherapy needle, or by employing endoscopic ultrasound. All injected animals survived and thrived without evidence of esophageal obstruction or perforation. There was some evidence that DxHA injection augmented the LES and result after injection. It should be emphasized that this is a small pilot study that does not conclusively offer data supporting a therapeutic role for DxHA in the treatment of GERT. The small sample size in each arm and the short survival period limit the power of the study. In addition, no animal model can duplicate the pathophysiology of human GERT. However, the data presented here continue to raise the possibility of a therapeutic role for this agent in GERT treatment in both children and adults. Further refinements in experimental techniques are necessary in order to produce stronger evidence and potentially allow for translational research in patients through a limited trial.

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References
