Advances in imaging and endoscopic therapy in Barrett's esophagus

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Steroid and botulin injection directly after extensive endoscopic resection do not prevent severe stenosis in an esophageal porcine model

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Abstract

Introduction: In the esophagus a high stenosis risk results from extensive endoscopic resection (ER), i.e. ≥3cm in length and ≥75% circumference. Steroids are used for treatment of benign stenosis, while botulin may prevent stenosis formation by relaxing the muscularis propria. Aim: to determine the efficacy of steroid and botulin injections immediately after extensive ER to prevent severe stenosis in a porcine model. Methods: A total of 8 pigs were included. In each pig 3 ER areas were created of 3cm in length and 75% circumference. Each of the 3 created wounds were injected after randomization with triamcinolone (40 mg), botulin (100 E), or saline. After 42 days all pigs were euthanized and esophagi were harvested for histological evaluation. Endpoints were number and severity of stenosis and depth of fibrosis in the esophageal wall. Results: Stenosis occurred in all ER areas injected with steroid, botulin or saline. The ratio of the circumference at the center and the upper edge of the ER area was not significantly different between the area injected with steroid 0.19 (±0.07), botulin 0.18 (±0.07) or saline 0.22 (±0.03). Fibrosis reached always the muscularis propria in areas injected with steroid or botulin and in 6/8 areas injected with saline. Fibrosis was seen transmurally in 2/8 ER areas injected with steroid, 1/8 injected with botulin and 0/8 injected with saline. Conclusions: Extensive ER results in deep fibrosis reaching the muscularis propria, which is probably the cause of severe stenosis. Steroid and botulin injections in the ER wound do not prevent stenosis formation after extensive ER in this porcine model.
Introduction

In the last decade endoscopic therapy has become the therapy of choice for esophageal cancer with limited invasion as it has been proven to be safe and effective.\textsuperscript{1,2} The cornerstone of endoscopic therapy is endoscopic resection which results in histological staging of the tumor and is therefore crucial for deciding adequate therapy according to the invasion depth of the tumor. Several endoscopic resection techniques are being used in the esophagus such as the cap technique, the multi-band mucosectomy technique or endoscopic submucosal dissection.\textsuperscript{3-13} An important drawback of endoscopic resection is the risk of stenosis. Stenosis rate is known to increase with the extent of the resected area in the esophagus. With resections comprising 75\% of the circumference or more, stenosis rate has been reported in up to 90\% of the patients.\textsuperscript{13-15} Stenosis reduces patient's quality of life and repetitive dilations are usually necessary. Another important consequence is that stenosis may hamper further endoscopic therapy that might be necessary for complete removal of neoplasia.

New methods to prevent stenosis after widespread resection are thus necessary. A possible strategy to prevent stenosis is by trying to interfere in the processes of wound healing. Normally, wound healing consists of three phases: inflammation, proliferation and remodeling. The inflammatory phase occurs quickly after the wound is created and persists during the first days. During the inflammatory phase cytokines are released and cells are recruited. Several days after the wound is created, the proliferation phase starts. During the proliferation phase epithelialization of the wound occurs in combination with fibroblast proliferation and concomitant production of collagens. After several weeks re-epithelialization is completed and remodeling starts by maturation of the collagen matrix, including collagen cross-linking. The remodeling phase continues for a prolonged time and can extend over a year.\textsuperscript{16} A possible method to interfere with the wound healing and prevent stenosis is the use of intralesional steroids. On the one hand steroids might prevent excessive inflammation and thereby excessive fibroblast proliferation and collagen production, and therefore stenosis formation. On the other hand steroids might inhibit inflammation necessary for re-epithelialization and healing, therefore giving problems in the healing process. In addition, steroids are believed to prevent collagen cross-linking during the remodeling phase.\textsuperscript{17} This might be the explanation why several studies have shown that injection of steroids into recurrent or refractory benign esophageal stenoses may improve the outcome after esophageal dilation.\textsuperscript{18-21} Another possible strategy to prevent stenosis formation after widespread endoscopic resection might be the use of botulin injections into the proper muscle layer. Botulin is a toxin that inhibits the release of acetylcholine in the nerve endings and therefore prevents the neuromuscular
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Impulse transmission resulting in muscle relaxation. Inhibition of this transmission is temporally and restores gradually in approximately twelve weeks as nerve endings make new contacts. Botulin injections in the proper muscle layer of the esophagus might thus result in temporary muscle relaxation. This muscle relaxation might impede contraction of the esophagus at the resection wound during the proliferation phase of the healing and therefore possibly preventing severe stenosis formation.

The aim of this study was therefore to determine the efficacy of steroid and botulin injections immediately after extensive endoscopic resection to prevent severe stenosis in a porcine model.

Methods

For the purpose of this study a porcine model was used consisting of a total 8 female pigs with a weight of 46-53 kg. Experiments were performed at the Animal Research Institute AMC (ARIA) after protocol approval by the Animal Ethical Committee at the Academic Medical Center in Amsterdam, Netherlands.

Animal handling

Animal care was in accordance with European Union guidelines. All pigs entered the animal facilities 13 days prior to the start of the experiments for acclimatizing purposes. Pigs received a semi liquid diet and were placed on a grid 2 days before the experiment and fasted with free access to water 16 hours before the experiment. For the experiment, pigs were sedated with intramuscular midazolam 1 mg/kg and ketamine 15 mg/kg, followed by endotracheal intubation. After induction with propofol 3 mg/kg, anesthesia was maintained with propofol 6mg/kg/h and supported by 2% isoflurane when necessary. Pigs were placed in left lateral and anti-Trendelburg position.

Endoscopic resection

Endoscopic resection was performed with the cap technique by using a hard oblique cap (12 mm diameter) attached to the tip of the endoscope. First, the treatment area was lifted with submucosal injection of diluted adrenaline (1:100.000 NaCl 0.9%) with an injection needle (Interject, Boston Scientific, Natick, Massachusetts). After placing the cap on the tip of the endoscope and prelooping a crescent shaped snare (EMR Kit, Olympus Europe, Hamburg, Germany) in the distal rim of the cap, the mucosa was sucked into the cap. By tightening the snare a pseudopolyp was created that was resected using 45 Watt pure coagulation current.
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(Erbotom ICC 200, ERBE Elektromedizin GmbH, Tuebingen, Germany). In case of subsequent resections in the treatment area, submucosal lifting was repeated and a new snare was used.

**Experiment**

For all procedures a therapeutic endoscope was used (GIF-1T140 Olympus, Tokyo, Japan). Food remnants in the stomach were removed with endoscopic suction and the esophagus was rinsed with water. In each pig 3 treatment areas were marked by placing electrocoagulation marks with the tip of the snare. Each treatment area was 3 cm long and included 75% of the circumference (Figure 1). Treatment areas were resected with the above described cap technique. Subsequently each resection wound was injected with one of the following substrates: triamcinolone, botulin toxin, or saline. Triamcinolone acetonide 40 mg (Kenacort-A ’40’ 1 ml ampoule, Bristol-Meyers Squibb, Princeton, New Jersey) was dissolved in 3 ml of saline 0.9% and 1 ml aliquots (10 mg) were injected into the submucosa at 4 locations in the resection wound. Botulin toxin 100 E (Botox, Allergan, Irvine, California) was dissolved in 4 ml of saline 0.9% and 0.5 ml aliquots (12.5 E) were injected into the muscularis propria at 8 locations in the resection wound. Saline (0.9%) was injected in 1 ml aliquots at 4 locations in the resection wound as control. For each solution separate injection needles (Interject, Boston Scientific, Natick, Massachusetts) and syringes were used. Treatment areas were randomized prior to injection resulting in: distal steroid, mid saline, proximal botulin; or distal botulin, mid saline, proximal steroid. At the end of the procedure, all treatment areas were marked just proximal with two tattoos (SPOT endoscopic marker, GI Supply, Camp Hill, Pennsylvania) and the pigs were detubated.

![Figure 1 Placement of markers prior to endoscopic resection of a treatment area (A). After endoscopic resection with the cap technique the resection wound was 3cm in length and contained 75% of the circumference (B).](image)
Follow-up

Pigs received a semi liquid diet and were placed on a grid for 3 days in order to prevent sawdust perforating the esophageal wounds. After 3 days pigs progressively received a more solid diet. In case of regurgitation, i.e. stenosis, pigs were offered again a semi liquid diet which was supplemented with milk protein if additional caloric intake was necessary. Pigs were euthanized with an intra venous overdose of pentobarbital after which the esophagus was harvested for histology 42 days after the experiment.

Histology

After harvesting the esophagus, the treatment areas were identified by opening the esophagus in longitudinal direction. The treatment areas were cut out and stretched on paraffin with pins (Figure 2). After stretching on paraffin and before fixation in formalin the mucosal circumference of each treatment area was measured with a ruler at the center of the treatment area and at the upper edge of the treatment area (Figure 3). To correct for anatomic differences in the macroscopic circumferential measurements, a ratio was calculated: the circumference of the center of the stenosis was divided by the circumference 2 cm proximal of the stenosis. After at least 24 hours fixation in 10% formalin solution each treatment area was cut into 4 mm slices resulting in several slices per treatment area. Each slice was embedded in paraffin and cut in 4 μm slides for standard haemotoxilin & eosin staining. Histological evaluation was performed by a gastro-intestinal expert pathologist (MV). Specimens were evaluated for the presence and depth of inflammation, fibrosis and necrosis. The deepest layer with damage due to inflammation and/or fibrosis was recorded.

Endpoints

Primary endpoint was the number and severity of stenosis after botulin, steroid or saline injection at 42 days. Secondary endpoint was the depth of fibrosis in the esophageal wall on histology after steroid, botulin or saline injection at 42 days.

Statistical analysis

Statistical analysis was performed with the Statistical Software Package version 16.0.2 for windows (SPSS, Chicago, Illinois, USA). For descriptive statistics, mean with standard deviation was used. Differences in the ratio of the circumference at the center and the upper edge of the endoscopic resection area were tested with the paired T-test.
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Figure 2 One of the harvested esophagi (A), opened in longitudinal direction showing the white mucosa (B): on the left the distal part on the right the proximal part. Treatment areas were separated and stretched on paraffin (C, E, F). Treatment areas were directly after endoscopic resection injected with botulin in the muscularis propria (C), saline (D) and steroid in the submucosa (E).

Figure 3 After opening an esophagus in longitudinal direction, treatment areas were separated and stretched on paraffin. Next, in each treatment area the circumference of the mucosa at the center of the stenosis (A) and the circumference of the mucosa 2 cm proximal of the stenosis (B) was measured in cm. To correct for anatomic differences a ratio of the center (A) to the proximal circumference (B) was calculated.

Results

Baseline characteristics

Eight female pigs started with a mean weight of 48 kg (range 43-53 kg) and ended with a mean weight of 74 kg (range 61-90 kg) after 42 days. During this experiment no acute or delayed perforations occurred.
Mean circumference of the untreated esophagus after 42 days was 8 cm when stretched, the calculated diameter based on this circumference was 25 mm. Mean circumference of the untreated proximal esophagus after 42 days was 8.5 cm when stretched, the calculated diameter based on this circumference was 27 mm. Mean circumference of the untreated mid esophagus after 42 days was 7.7 cm when stretched, the calculated diameter based on this circumference was 25 mm. Mean circumference of the untreated distal esophagus after 42 days was 7.9 cm when stretched, the calculated diameter based on this circumference was 25 mm.

**Stenosis rate**

Symptoms of regurgitation were first observed after a mean of 12 days (range 4-28 days). Severe stenosis occurred in all pigs in all endoscopic resection areas injected with steroid, botulin or saline after 42 days (Figure 2). Mean circumference of the endoscopic resection areas after 42 days when stretched was 1.5 cm after steroids injection, 1.4 cm after botulin injection and 1.7 cm after saline injection (Table 1). Mean calculated diameter based on this circumference was 5 mm after steroids injection, 4 mm after botulin injection and 5 mm after saline injection. The ratio of the circumference at the center and the upper edge of the endoscopic resection area was not significantly different between the areas injected with steroids (0.19), botulin (0.18) or saline (0.22) (Table 1).

**Table 1.** Severity of stenosis for botulin, steroid and saline injection directly after extensive endoscopic resection.

<table>
<thead>
<tr>
<th></th>
<th>Mucosal circumference</th>
<th>Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm (±SD)</td>
<td>center/proximal (±SD)</td>
<td></td>
</tr>
<tr>
<td><strong>Steroid injection</strong></td>
<td>1.5 (±0.5)</td>
<td>0.19 (±0.07)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Botulin injection</strong></td>
<td>1.4 (±0.5)</td>
<td>0.18 (±0.07)</td>
<td></td>
</tr>
<tr>
<td><strong>Saline injection</strong></td>
<td>1.7 (±0.2)</td>
<td>0.22 (±0.03)</td>
<td></td>
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</tbody>
</table>

*SD* standard deviation.

**Depth of fibrosis in esophageal wall**

Fibrosis reached always the muscularis propria in endoscopic resection areas injected with steroid or botulin and in 6 of the 8 endoscopic resection areas injected with saline. Fibrosis was seen transmurally (*i.e.* in all layers of the esophagus and through the complete muscularis propria) in 2 of the 8 endoscopic resection areas injected with steroid, 1 of the 8 endoscopic resection areas injected with botulin and none of the 8 endoscopic resection areas injected with saline (Table 2).
Table 2. Deepest layer with fibrosis 42 days after extensive endoscopic resection directly injected with botulin, steroid or saline.

<table>
<thead>
<tr>
<th></th>
<th>Steroids</th>
<th>Botulin</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submucosa</td>
<td>-</td>
<td>-</td>
<td>25% (2/8)</td>
</tr>
<tr>
<td>Muscularis propria</td>
<td>75% (6/8)</td>
<td>88% (7/8)</td>
<td>75% (6/8)</td>
</tr>
<tr>
<td>Transmural</td>
<td>25% (2/8)</td>
<td>12% (1/8)</td>
<td>0% (0/8)</td>
</tr>
</tbody>
</table>

Discussion

Although steroid and botulin can hypothetically prevent stenosis, steroid injection into the submucosa and botulin injection into the muscularis propria directly after endoscopic resection do not prevent stenosis formation after extensive endoscopic resection in this porcine model. Extensive endoscopic resection results in deep fibrosis reaching the muscularis propria, which is probably the cause of severe stenosis.

Stenosis was severe in all endoscopic resection areas treated directly with submucosal steroid (triamcinolone 40mg) injection. Others have also reported no preventive effect on stenosis formation of triamcinolone 80mg injection directly after endoscopic resection in a porcine model. In addition, peri-esophageal abscess formation was observed in these pigs, while in our study no complications were observed. Reports in patients on the preventive effect on stenosis after endoscopic resection are more promising as stenosis is only seen in 5-19% of the patients that underwent extensive (>75% of the circumference) endoscopic resection followed by some form of steroid treatment combined with dilation in case of dysphagia. Apart from the fact that we used a porcine model without performing dilations, the human studies used higher doses (100mg triamcinolone), repeated injections, and oral administration. It might therefore be that the dose of 40mg triamcinolone we used is too low or was not administrated at the most optimal moment.

The timing of steroid administration is probably important. As mentioned in the introduction healing consists of three different phases. Although it seems logical to administer steroids directly after endoscopic resection in order to have a maximum inhibiting effect on inflammation, it might be more effective to inhibit the healing at another phase. By injecting steroids several days after endoscopic resection the proliferation phase might be inhibited more optimal and thereby the fibroblast proliferation and collagen production. Inhibiting the inflammation phase as well the proliferation phase might have even an additional effect on inhibiting fibrosis formation and therefore stenosis. Hashimoto et al injected steroids several days after endoscopic resection, at day 3, 7 and 10. Also Yamaguchi et al started 3 days after endoscopic resection but prolonged the oral steroids for 8 weeks. In our experiment we did not perform the repeated steroid
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injections as repeated anesthesia was necessary which would lead in our opinion to an unethical burden for the pigs.

In this study fibrosis reached always the muscularis propria after steroid or botulin injection. This effect seems not to be related to steroid or botulin as even in the control areas fibrosis reached the muscularis propria in the majority of cases. Furthermore, during the endoscopic procedure the muscularis propria never appeared damaged. Fibrosis reaching the muscularis propria after endoscopic resection has been observed in dogs as well.26 A possible explanation might be that the coagulation current of the snare damages deeper layers that appear not damaged directly after endoscopic resection. Another possible explanation might be that inflammation during healing invades the muscularis propria destroying its fibers which as a consequence are replaced by fibrosis.

The major limitation of this study is that a porcine esophagus is not completely comparable to a human esophagus. Although all layers present in a human esophagus are present in the porcine esophagus, the layers are much thinner. In addition, the muscularis mucosae is almost as thick as the muscularis propria in the distal part of the porcine esophagus, while the proximal part has almost no muscularis mucosae and a very thick submucosa. It might be possible that due to these differences in the esophageal wall architecture, stenosis is formed more severely in pigs therefore reducing the effect of botulin and steroid to a minimum that is not appreciated in the low number of pigs used in this experiment. Nevertheless, we can exclude a large preventive effect on stenosis from triamcinolone 40mg and botulin 100E injected directly after extensive endoscopic resection in our porcine model.

Although this study did not show any preventive effect on stenosis formation of submucosal steroid injection directly after endoscopic resection, it seems still valuable to explore strategies that interfere with the process of wound healing in order to prevent stenosis formation. Not only because it seems plausible that with inhibition of inflammation fibroblast proliferation and collagen production will be reduced and therefore stenosis formation can be prevented, but also because results in patients are promising and encourage to evaluate this strategy further.23-25 Besides steroids, another possible way of inhibiting excessive inflammation and therefore fibrosis might be the use of non-steroidal anti-inflammatory drugs. For example in rats, stenoses after caustic ingestion were less severe when ibuprofen was administrated.27 Other strategies that might prevent stenosis formation after endoscopic resection are also being explored. Recently, endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial cell sheets was applied in nine patients after widespread endoscopic resection and stenosis only developed in one patient.28 Another strategy is the use of an extracellular matrix scaffold placed endoscopically
directly after endoscopic resection that prevented esophageal stricture formation in dogs. In conclusion, steroid directly injected in the submucosa and botulin toxin injection into the proper muscle layer after endoscopic resection do not prevent the stenosis formation after extensive endoscopic resection in this porcine model. Furthermore, extensive endoscopic resection results in deep fibrosis reaching the muscularis propria, which is probably the cause of severe stenosis. Other therapies preventing excessive fibrosis and therefore stenosis after extensive endoscopic resection still need to be explored.

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References


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