Topical Mithramycin-A Modulates Submucosal Collagen Deposition after Esophageal Burn Injury in Rats
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What is This?
Abstract

Objective. To evaluate efficacy of a drug-eluting, dissolvable esophageal (DEDE) stent for the prevention of submucosal collagen deposition in a rat model of acute esophageal injury.

Setting. University laboratory.

Study Design. Interventional randomized controlled trial.

Subjects and Methods. Forty two adult, male, age-matched Sprague Dawley rats were randomized to undergo either sham esophageal surgery, esophageal burn injury, or esophageal burn injury and placement of a DEDE stent. All animals underwent open gastrotomy under anesthesia. In group 1, a cautery device was inserted through the gastrotomy into the distal esophagus and removed without creating an injury. In group 2, the cautery was placed in the distal esophagus and a circumferential thermal burn injury was created. In group 3, an identical burn injury was created and a DEDE stent was placed at the site of injury and secured. On postoperative day 28, animals were sacrificed, and the distal esophagi were harvested and processed for histology. Submucosal collagen area was quantified histologically and compared across the 3 experimental groups.

Results. After the investigators controlled for luminal circumference and multiple measurements, submucosal collagen area was increased in group 2 (burn) compared with group 1 (sham) \((P = .012)\). Submucosal collagen area was decreased in group 3 (DEDE stent) compared with group 2 \((P = .042)\). No statistically significant difference in submucosal collagen area was observed between animals in group 1 and group 3 \((P = .800)\).

Conclusions. Topical application of mithramycin-A via a DEDE stent modulates collagen deposition after acute thermal injury in the rat esophagus.

Keywords

esophagus, stricture, collagen, mithramycin-A

The incidence of benign esophageal stricture disease is estimated to be 1/10,000, and the disease affects up to 375,000 Americans.\(^1\) It is associated with significant morbidity attributable to dysphagia and associated therapeutic interventions.\(^2\) Causes include gastroesophageal reflux disease, radiation therapy, trauma, caustic ingestion, autoimmune diseases, and idiopathic conditions.\(^3\) Stricture is a direct result of mucosal damage followed by subepithelial collagen deposition.\(^4\) Healing from this injury occurs in phases beginning with acute inflammation, followed by fibroplasia (collagen deposition), and concluding in maturation (contraction). This process does not replace injured tissue by multi–germ layer regeneration but rather fills the defect with scar tissue that reduces esophageal distensibility and compliance.\(^4,5\) Because of the tubular nature of the esophagus, scar contraction proceeds circumferentially leading to esophageal stenosis with resultant dysphagia, malnutrition, pain, stomach tube dependence, and reduced quality of life.\(^6\)

The current treatment paradigm for esophageal stricture is repeated endoscopic dilatation—an unfortunate cycle of mucosal disruption and scar reformation. Recognition that the synthesis, deposition, and remodeling of collagen are the fundamental processes underlying stricture formation has led to experimentation with a variety of pharmacologic agents in small animal models of esophageal stricture.\(^5,7-14\)

To modulate collagen deposition during the healing process, we sought to test the topical application of mithramycin-A (MMA) directly at the site of an acute esophageal injury in a rodent model. Mithramycin-A is an antiproliferative agent that
has been used to treat testicular cancer. Recently, MMA has been shown to specifically inhibit fibroblastic synthesis of type 1 collagen. When administered systemically as an antineoplastic agent, MMA has demonstrated high toxicity. At nanomolar concentrations, however, the collagen gene inhibition is specific and the antiproliferative cellular effects of large systemic doses are minimized. In this experiment, a bioabsorbable polyactic–glycolic acid (PLGA) esophageal stent was fashioned and loaded with MMA to create a drug-eluting, dissolvable esophageal (DEDE) stent for local/topical application of drug at the site of esophageal injury. In this study, we aimed to determine whether such a method of local/topical drug delivery would ameliorate subepithelial collagen deposition in a rat model of acute esophageal injury.

**Methods**

**Animals**

This study was approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. Forty-two 10-week-old, adult, male wild-type Sprague Dawley rats were individually housed in standard rodent cages. Ten animals were randomized to the sham surgery group (group 1), 16 animals were randomized to the burn-only group (group 2), and the remaining 16 animals were randomized to the burn and stent group (group 3). Animals had free access to water throughout the study. All animals had access to ad libitum amounts of DietGel (ClearH2O, Portland, Maine) for the first postoperative week. During week 2 of the study, animals were fed wet chow. During weeks 3 and 4, the animals were fed normal rat chow. Animals were weighed weekly.

**Stent Fabrication**

We fabricated D,L-lactide-co-glycolide (PLGA) esophageal stents using an electrospinning technique at 900 revolutions per minute. We prepared PLGA (molecular weight 71 kDa) as a solution of 50% polyglycolic acid and 50% polylactic acid by weight. We fashioned 10-mm tubular stents with an internal diameter of 3.0 mm and an outer diameter of 3.2 mm, and 500 µg of MMA powder (AG Scientific, San Diego, California) was incorporated into the prespinning solution of each stent (Figure 1).

**Scanning Electron Microscopy**

Stents were fixed in osmium tetroxide (2%) and sequentially dried in ethanol. Specimens were mounted and coated with gold alloy, stored in a desiccator for 24 hours, and then examined by scanning electron microscopy (Philips FEI quanta200, Hillsboro, Oregon).

**In Vitro Drug Elution**

Drug elution was performed by placing 3 specimens in phosphate-buffered saline (PBS) on a shaker at 37°C. Next, 500-µL samples of solution were drawn off at 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours, and then daily for 50 days. Specimens were transferred into fresh PBS solution at each time interval, and drug levels were measured using a spectrophotometer at 275 nm.
Tissue Preparation
The distal esophagus was identified and separated from the stomach. The specimens were fixed in formalin for 24 hours. Afterward, they were processed and embedded in paraffin.

Histopathologic Evaluation
Distal esophageal specimens were sectioned at a thickness of 4 µm. Ten representative sections over a 400-µm length of tissue were selected for evaluation. Alternating sections were stained with either trichrome collagen stain (Masson Trichrome Kit, Sigma-Aldrich, St. Louis, Missouri) or hematoxylin and eosin. Low-power light microscopy was used to evaluate the lumen circumference (in micrometers). The cross-sectional area of the subepithelial collagen layer was calculated (in square micrometers) using ImagePro (version 6.3; Media Cybernetics, Inc, Bethesda, Maryland) (Figure 4).

Statistical Analyses
Data are presented as mean ± standard deviation of the mean. One-factor analysis of variance was used for comparisons of normally distributed continuous data among the 3 treatment groups. Model-based means were compared using a mixed-model analysis of variance, adjusted for repeated measurements and luminal circumference. Post hoc comparisons between experimental groups were evaluated using the Tukey method for adjusted P values. Data were analyzed using SAS for Windows (version 9.2; SAS Institute Inc, Cary, North Carolina). P < .05 was considered significant.

Results
Surgical Model
Forty-two animals (100%) survived surgical interventions; 39 animals survived until the study end point. Three of the ani-
mals in group 3 (burn with DEDE stent) died within 3 days postoperatively because of esophageal obstruction and inanition. There was no gross evidence of granulation or epithelial hyperplasia in the distal esophagus when it was harvested at the study end point.

Animal Weights
All experimental animals gained weight over the study time course. In group 1 (sham surgery), weight gains ranged from 39.6 to 137.1 g (mean 72.8 g). Animals in group 2 (burn only) demonstrated weight gains ranging from 22.1 to 94.2 g (mean 59.3 g). In group 3 (burn and DEDE stent), weight gains ranged from 25.8 to 111.1 g (mean 65.6 g). There was no statistically significant difference in weight means across groups at the study end point ($P = .24$).

Verification of MMA Loading on Stents
Stents incorporating MMA were grossly yellow in color. Images from scanning electron microscopy showed webbing and presence of MMA between fibers on drug-loaded stents. In vitro drug elution from the MMA stents demonstrated a 2-phase release profile with an initial burst release in the first 24 to 48 hours with a maximum drug concentration of $1 \times 10^{-5}$ mol/L. Thereafter, a stable release profile was observed over 1176 hours (approximately 48 days) that ranged from $3.67 \times 10^{-6}$ to $5.08 \times 10^{-6}$ mol/L (Figure 5).

Stent Degradation
The PLGA tents degraded completely over 63 days. The most favorable degradation pattern was demonstrated at pH 7 with no stent fragmentation until after 48 days. Luminal collapse occurred at 39 days at pH 5 and at 48 days at pH 6 and pH 7.

Submucosal Collagen Measurements
The adjusted mean submucosal collagen area for group 1 (sham, $n = 16$) was 386,086 µm² (range, 354,661-417,510 µm²; standard deviation 15,495 µm²). The adjusted mean submucosal collagen area for group 2 (burn only, $n = 10$) was 545,956 µm² (range, 446,833-645,079 µm²; standard deviation 48,875 µm²). The adjusted mean submucosal collagen area for group 3 (burn and MMA stent, $n = 13$) was 408,673 µm² (range, 343,782-473,564 µm²; standard deviation 31,996 µm²). After we controlled for luminal circumference and repeated measures, submucosal collagen area was increased

Figure 5. Representative in vitro drug release profile for mithramycin-A (MMA)–loaded stents. Molar concentrations of MMA ranged from $1 \times 10^{-5}$ mol/L at 24 hours to $3.86 \times 10^{-6}$ mol/L at 1176 hours (48 days). There was an initial burst of drug in the first 24 to 48 hours and then a steady release of drug persisted for 7 weeks.

Figure 6. Representative trichrome-stained 4-µm sections of control, burn-only, and burn + mithramycin-A stent experimental groups ($\times$10 magnification). Note the markedly increased submucosal collagen (blue-green in color) in burn specimen (center) compared with control (left) and burn + stent (right) specimens. Note the similarity in submucosal collagen staining between the sections from stented and control specimens.
Sp1 appears to act specifically on the transcription factor the scar tissue found in esophageal stricture. Mithramycin-A is an anticancer antibiotic derived from the Streptomyces, previously used to treat testicular cancer as well as intractable hypercalcemia, this drug has recently been identified as a modulator of type 1 collagen. Type 1 collagen, consisting of two α1 chains and one α2 chain, is a principal component of the scar tissue found in esophageal stricture. Mithramycin-A appears to act specifically on the transcription factor Sp1. In turn, Sp1 is responsible for expression of the promoter for the α1 chain of type 1 collagen (COL1A1).

Figure 7. Comparison of submucosal collagen area across groups, adjusted for lumen circumference and repeated measures. In group 1 (sham surgery), the adjusted mean equaled 386,086 µm² with a standard deviation of 15,495 µm. In group 2 (burn only), the adjusted mean equaled 545,956 µm² with a standard deviation of 48,875 µm. In group 3 (burn and MMA stent), the adjusted mean equaled 408,673 µm² with a standard deviation of 31,996 µm.

in group 2 compared with group 1 (P = .012). Submucosal collagen area was decreased in group 3 compared with group 2 (P = .042). No difference was observed between animals in group 1 and group 3 (P = .800) (Figures 6 and 7).

Discussion
Submucosal collagen deposition in the esophageal wall has long been recognized as the major contributor to fibrosis associated with esophageal stricture independent of cause. A variety of pharmaceutical agents, including N-acetylcysteine, mitomycin, halofuginone, sphingosylphosphorylcholine, penicillamine, ketotifen, and corticosteroids, have been used in attempts to alter this process. In animal models, these agents have been administered enterally, applied by esophageal gavage, and administered systemically in the form of intraperitoneal injection. Systemic administration of anti-inflammatory agents and antiproliferatives is nonspecific and dose limited because of potential drug side effects. Topical application of a collagen gene inhibitor such as MMA has advantages. First, it is specific to the underlying pathophysiology of stricture formation. Second, it exerts its therapeutic effect at the site of injury and therefore decreases the amount of drug necessary for therapeutic effect. To test the concept of local drug delivery, a drug-eluting dissolvable esophageal stent was developed for use in an animal model of acute thermal esophageal mucosal injury.

Stenting of the esophagus in humans with nonbioabsorbable materials has been largely unsuccessful in the treatment of benign stricture. These stents have high failure rates and must often be removed because of stent migration, mechanical failure, or granulation tissue. A stent made of PLGA is readily hydrolyzed and breaks down into lactic acid and water.

To deliver MMA precisely to the site of injury, it was necessary to devise an animal model in which esophageal injury could be discretely located and identified both at the time of tissue inspection and at analysis. Animal models for esophageal stricture are well established using a “caustic stricture” approach to injury. Exposure to a strong base produces a significant amount of scarring and mural injury. This model does not entirely represent the treatment paradigm for the more common clinical picture of a short segment esophageal stricture that is noncaustic in etiology. For purposes of this study, the main problem with applying a strong alkali to the esophagus to create a burn was the potential alteration in pH and the unknown effect that this might have on MMA activity and possibly even stent degradation kinetics. For all of these reasons, the animal model protocol devised for this study was developed with anticipation of a clinical application in which an established short segment stricture (eg, radiation, gastro-esophageal reflux disease, surgical) is dilated and treated immediately with a topical or locally injectable agent such as is commonplace in clinical practice.

With regard to use of a dissolvable stent in a rat, no prior experimental literature exists. Stenting of the esophagus in humans with nonbioabsorbable materials has been largely unsuccessful in the treatment of benign stricture. These stents have high failure rates and must often be removed because of stent migration, mechanical failure, or granulation tissue. A bioabsorbable stent that can be safely metabolized or eliminated by the body would provide a notable improvement in this aspect of the treatment paradigm for esophageal stenosis. A stent made of PLGA is readily hydrolyzed and breaks down into lactic acid and water.

In this study, complete in vitro stent degradation was predictable at pH 7 (the typical pH of the rat esophagus). In vivo, all but 2 stents had dissolved at 28 days. The 2 stents that remained were partially degraded, and there was no gross evidence of epithelial hyperplasia or granulation in the distal esophagus of any of the 39 study animals at autopsy. These data appear consistent with the assumption that stents would degrade faster in vivo because of the presence of salivary amylase activity on the carbohydrate moiety of the PLGA substrate. Three of the group 3 animals died within 3 days postoperatively. Autopsy demonstrated no evidence of esophageal perforation but did demonstrate bed-infarctions in the esophagus proximal to the stent that had remained in position. Review of the literature suggested that these animals were demonstrating pica, a common and
well-described behavior in rodents during periods of gastrointestinal distress. Accordingly, upon recommendations of veterinary staff, all animals in groups 2 and 3 were kept in identical wire-bottom cages for the first 2 weeks of the study and fed a high-calorie gel diet for the first postoperative week. With these changes, stents were well tolerated in the remaining animals as evidenced by mean weight change data. Although not statistically significant, the trend in mean weight change over the study period (sham surgery animals gained the most weight, burn-only animals gained the least, DEDE stent animals gained an intermediate amount) further supports the feasibility of additional research using a dissolvable drug-eluting stent in a rat model.

The mechanism and rate of drug release are closely tied to the composition of the PLGA stent. As the PLGA substrate breaks down into lactic acid and water, drug is released (eluted) from the stent. In vitro, stent drug elution data verified the presence of MMA at molar concentrations within the reported range for specific COL1A1 inhibition in a fibroblast cell culture system. In vivo, submucosal collagen deposition was reduced in DEDE-stented animals (P < .05) compared with burn-only animals, suggesting that a sufficient amount of drug was delivered at the site of burn injury to reduce collagen deposition. Because rats, as a species, are incapable of emesis and because the full length of bowel was examined at sacrifice, it can be concluded that stents remained in place long enough to deliver drug to the site of injury, and therefore reduced submucosal collagen was attributed to drug effect. A weakness of this study is that the presence of MMA in serum (systemic) was not verified. In preliminary studies (unpublished data) using readily available methods (spectrophotometry), we were unable to confirm the presence of drug in serum because of similar absorbance wavelengths of serum proteins and MMA. High-pressure liquid chromatography would have been necessary to identify drug in serum, and this was beyond the resources of this investigation. Quantification of collagen gene expression by polymerase chain reaction in tissue could further test the feasibility of dissolvable esophageal stents as well as the efficacy and safety of MMA as a potential treatment adjunct in the management of esophageal stricture.

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Author Contributions
Paul Lawson Davis III, study conception and design, analysis and interpretation of data, drafting and revising of article; Scott Hardison, study design, analysis and interpretation of data; Christopher A. Sullivan, study conception and design, analysis and interpretation of data, drafting and revising of article, final approval of published version.

Disclosures
Competing interests: Christopher A. Sullivan is an inventor on a patent application for the technology presented in this manuscript. Wake Forest University Health Sciences has licensed this technology to Applied Catheter Technologies, Inc. (ACT). Wake Forest University Health Sciences and Dr Sullivan have the potential to receive income related to this invention if ACT should develop this technology. ACT did not fund any of this research and did not participate in study conception, design, study conduct; data collection, analysis and/or interpretation; writing or approval of the manuscript.

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