RESEARCH ARTICLE

Bilayer silk fibroin grafts support functional oesophageal repair in a rodent model of caustic injury

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Abstract
Surgical repair of caustic oesophageal injuries with autologous gastrointestinal segments is often associated with dysmotility, dysphagia and donor site morbidity, and therefore alternative graft options are needed. Bilayer silk fibroin (BLSF) scaffolds were assessed for their ability to support functional restoration of damaged oesophageal tissues in a rat model of onlay oesophagoplasty. Transient exposure of isolated oesophageal segments with 40% NaOH led to corrosive oesophagitis and a 91% reduction in the luminal cross-sectional area of damaged sites. Oesophageal repair with BLSF matrices was performed in injured rats (n = 27) as well as a nondiseased cohort (n = 12) for up to 2 months after implantation. Both implant groups exhibited >80% survival rates, displayed similar degrees of weight gain, and were capable of solid food consumption following a 3-day liquid diet. End-point u-computed tomography of repaired sites demonstrated a 4.5-fold increase in luminal cross-sectional area over baseline injury levels. Reconstructed oesophageal conduits from damaged and nondiseased animals produced comparable contractile responses to KCl and electric field stimulation while isoproterenol generated similar tissue relaxation responses. Histological and immunohistochemical evaluations of neotissues from both implant groups showed formation of a stratified, squamous epithelium with robust cytokeratin expression as well as skeletal and smooth muscle layers positive for contractile protein expression. In addition, synaptophysin positive neuronal junctions and vessels lined with CD31 positive endothelial cells were also observed at graft sites in each setting. These results provide preclinical validation for the use of BLSF scaffolds in reconstructive strategies for oesophageal repair following caustic injury.

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1 | INTRODUCTION

Accidental ingestion of caustic alkali agents is highly prevalent in the worldwide paediatric population and frequently results in corrosive oesophagitis, stenosis, and ultimately oesophageal stricture formation (Uygun, 2015). Endoscopic balloon dilatation and the use of temporary stents represent the primary treatment options for increasing luminal oesophageal calibre while also improving dysphagia symptoms associated with caustic injury (Dall’Oglio et al., 2016). Unfortunately, dilatation of the oesophagus carries the risk of organ perforation and, over time, this pathology can become refractory to this approach (Ramareddy & Alladi, 2016). Similarly, stent-related complications such as device migration and erosion are significant concerns for this mode of therapy (Zhao, Zhou, Feng, Wang, & Mei, 2016). In patients where conventional management fails, gastric transposition and colonic interposition grafts are used to replace diseased oesophageal segments (Ezemma, Eze, Nwafor, Etukokwu, & Orakwe, 2014). However, these procedures can lead to severe adverse events such as oesophageal dysmotility, anastomotic leakage and donor site morbidity, all of which can negatively impact patient quality of life (Reinberg, 2016). Matrices derived from decellularized tissues or synthetic polymers have been previously investigated as alternatives to autologous gastrointestinal segments in both animal models and clinical settings of oesophageal reconstruction (Aikawa et al., 2013; Badylak et al., 2011; Badylak, Meurling, Chen, Spievack, & Simmons-Byrd, 2000; Dua, Hogan, Aadam, & Gasparri, 2016). Suboptimal outcomes with these scaffold configurations including implant contracture, graft perforation and stenosis have been reported (Badylak et al., 2011; Doede,
Bondartschuk, Joerck, Schulze, & Goernig, 2009; Lopes et al., 2006), emphasizing the need to explore new biomaterials for oesophageal repair.

Bilayer silk fibroin (BLSF) grafts represent emerging, biodegradable platforms for oesophageal tissue engineering. The multifunctional implant design promotes initial defect consolidation and preservation of organ continuity via a fluid-tight film layer, whereas a porous foam compartment serves as a conduit for host tissue integration (Algarrahi et al., 2015; Seth et al., 2013). A recent report from our laboratory has demonstrated the feasibility of these matrices for onlay oesophagoplasty in a nondiseased, rodent model of acute traumatic injury (Algarrahi et al., 2015). In this system, BLSF scaffolds promoted constructive remodelling of oesophageal defects with neotissues capable of supporting peristalsis and solid food consumption (Algarrahi et al., 2015). Parallel comparisons with conventional small intestinal submucosa (SIS) grafts revealed BLSF matrices achieved significantly higher degrees of skeletal muscle formation, de novo innervation, as well as reduced inflammatory reactions within implantation sites (Algarrahi et al., 2015).

Although the initial performance of BLSF scaffolds for oesophageal repair was encouraging, the use of nondiseased animal models may not accurately predict graft performance in patients with underlying pathologies due to alterations in the regenerative capacity of host tissues. For instance, Akbal et al. (2006) reported that while augmentation of healthy porcine bladders with an acellular dermal biomatrix resulted in excellent functional bladder tissue regeneration, similar experiments in a porcine model of obstructed bladder disease failed to show favourable results. Moreover, onlay urethroplasty of damaged rabbit urethras with SIS grafts revealed delayed epithelialization and abnormal distribution of smooth muscle tissue in comparison to the outcomes achieved in healthy animals (Villoldo et al., 2013). Therefore, the objective of this study in rats was to establish whether BLSF scaffolds have the same regenerative capacity when onlay oesophagoplasty is performed following caustic injury in comparison to a nondiseased setting.

2 MATERIALS AND METHODS

2.1 Biomaterials

BLSF scaffolds were fabricated from Bombyx mori silkworm cocoons using a solvent-casting/salt-leaching process in combination with silk fibroin film casting as previously reported (Seth et al., 2013). The structural and tensile properties of the graft have been reported in published studies (Seth et al., 2013). Matrices were sterilized in 70% ethanol and rinsed in phosphate-buffered saline (PBS) prior to surgical procedures.

2.2 Surgical procedures

All animal studies were approved by the Boston Children's Hospital Animal Care and Use Committee prior to experimentation and performed under protocol 16-05-31611R.

Caustic oesophageal injury was induced in female Sprague-Dawley rats (n = 68, age 6–8 weeks, weight ~ 250–300 g, Charles River Laboratories, Wilmington, MA, USA) using a NaOH burn model described by Okata, Hisamatsu, Hasegawa, Nishijima, and Okita (2011) with some modifications. Prior to surgery, rats were maintained for a maximum of 24 h on a liquid diet consisting of a nutritionally-balanced commercial formula (TestDiet®, Richmond, IN, USA; mixed with Pediasure®; Abbott Laboratories, Columbus, OH, USA) and were given ready access to water. Under general anaesthesia induced by isoflurane inhalation, rats were placed in a supine position with the thorax elevated to 30°. An upper midline laparotomy incision was made through the skin and underlying rectus muscle in a sterile fashion in order to isolate a 1 cm segment of the abdominal oesophagus. A 5-French double lumen Vascu-PICC line (Medcomp®, Harleysville, PA, USA) was placed in the upper part of the abdominal oesophagus via the mouth and vessel loops (Devon™, Mansfield, MA, USA) were tied externally around the gastroesophageal junction and 5 mm proximal to compartmentalize NaOH exposure. Catheter infusion of a 40% NaOH solution was performed into the isolated oesophageal segment with a total contact time of 2 min which was followed by irrigation with distilled water and subsequent fluid aspiration. The catheter and vessel loops were then removed and non-absorbable 7–0 polypropylene sutures with 4–0 steel rings were positioned at the proximal/distal boundaries of the injured segment for region identification during imaging analyses described below. Following these procedures, the oesophagus was replaced into the abdominal cavity. The skin and abdominal incisions were sutured closed. Postoperative pain was managed with subcutaneous injection of 1 mg/kg meloxicam and 0.1 mg/kg buprenorphine. Rats were maintained on liquid diet and imaging evaluations were performed 2 days postinjury to evaluate luminal oesophageal calibre. Animals were then either harvested for 2 days postinjury analyses (n = 20), subjected to scaffold implantation (n = 27) as detailed below, or maintained without surgical intervention (n = 5).

Onlay oesophagoplasty with BLSF scaffolds was performed as previously described (Algarrahi et al., 2015) in rats at 3 days postinjury. In parallel, a cohort of nondiseased rats (n = 12) were subjected to oesophageal reconstruction with BLSF grafts. In both experimental groups, an upper midline laparotomy incision was steriley performed under isoflurane anaesthesia to expose the abdominal oesophagus. A 7 × 3 mm² elliptical defect was created in the anterior oesophageal wall 5 mm above the gastroesophageal junction via surgical tissue resection. An elliptical graft of equal size was anastomosed into the defect site using interrupted 7–0 polyglactin sutures. Nonabsorbable 7–0 polypropylene sutures were placed at the proximal/distal and lateral edges of the graft perimeter for identification of graft borders. The oesophagus was then returned to the abdominal cavity and incisions were sutured closed. Postoperative pain was managed as described above and rats were maintained on the aforementioned liquid diet for 3 days postoperatively and subsequently nourished with standard rat feed for the study duration. Animals were weighed prior to surgery and every week until scheduled euthanasia. Rats subjected to caustic injury and implanted with BLSF scaffolds were harvested for endpoint evaluations at 1 week (n = 5), 1 months (n = 5), and 2 months (n = 17) postrepair. All nondiseased animals grafted with BLSF scaffolds were harvested at 2 months postimplantation for comparative
analyses. An additional group of nonsurgical, healthy animals (n = 12) were evaluated in parallel as positive controls.

2.3 | Micro-computed tomography

Micro-computed tomography (µ-CT) analysis was performed on experimental groups following contrast agent gavage as previously described (Algarrahi et al., 2015). Luminal oesophageal cross-sectional areas (n = 7 animals per group) were quantified from the central regions of the original caustic injury sites 2 d following NaOH administration, scaffold implantation sites from damaged and nondiseased cohorts 2 months postrepair, as well as internal noninjured reference points adjacent to the seventh thoracic vertebra (T7) using published methods (Algarrahi et al., 2015). Parallel measurements were executed in corresponding oesophageal regions in nonsurgical controls.

2.4 | Ex vivo contraction and relaxation responses

Circular oesophageal tissues with intact mucosa (n = 5 animals per group) were isolated from injured sites 2 days after NaOH exposure, repaired segments from nondiseased and diseased groups at 2 months postoperatively, as well as nonsurgical controls. Specimens were mounted in tissue baths for isometric tension studies as previously described (Algarrahi et al., 2015). Briefly, contractile responses to KCl (80 mM) and electrical field stimulation (EFS; 25–20 Hz, 0.5 ms pulse duration, 40 V, 10 s) were measured. In parallel, specimens were precontracted with carbachol (3 μM) and relaxation responses were quantified following administration of isoproterenol (10 μM). Contractile responses were expressed as force (mN) normalized by tissue cross-sectional area. Relaxation responses were expressed as percent change from precontraction response.

2.5 | Histological, immunohistochemical and histomorphometric analyses

Following animal harvest, tubular oesophageal specimens isolated from experimental groups were formalin-fixed, dehydrated in graded alcohols, and paraffin embedded. Sections (5 μm) were stained with Masson’s trichrome (MTS) using standard methods. Parallel specimens were analysed for IHC assessments using primary antibodies to the following markers as previously described (Algarrahi et al., 2015): fast myosin skeletal heavy chain (MYH), α-smooth muscle actin (α-SMA), pan-cytokeratin (CK), CK4, CK14, filaggrin, synaptophysin (SYP) and CD31. In addition, host tissue responses were evaluated with the following primary antibodies: anti-myeloperoxidase (Abcam, Cambridge, MA, 1:100 dilution) and anti-CD68 (Thermo Fisher Scientific, Cambridge, MA, 1:200 dilution). Specimens were then stained with species-matched Alexa Fluor 488, 594, and 647-conjugated secondary antibodies (Thermo Fisher Scientific) while 4’6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. An Axiosplan-2 microscope (Carl Zeiss Microlmaging, Thornwood, NY, USA) was utilized for sample visualization and representative fields were acquired with Axiosvision software (version 4.8).

Histomorphometric evaluations (n = 4–7 animals per group) were performed using published methods (Algarrahi et al., 2015). Briefly, image thresholding and area measurements were acquired with ImageJ software (version 1.47) on four independent microscopic fields per tissue specimen (20× magnification) in order to calculate the percentage of tissue area stained for MYH, α-SMA and pan-CK per total tissue area examined. The number of SYP+ bountons were also quantified across four independent microscopic fields per tissue sample (20× magnification) employing similar methods and normalized to total tissue area analysed to determine density of synaptic transmission areas. Vessel density was determined in each tissue sample by normalizing the total number of CD31+ vessels present in two independent microscopic fields (5×) per total tissue area examined.

2.6 | Statistical analysis

Quantitative measurements were evaluated with the Kruskal–Wallis test in combination with the posthoc Scheffé’s method utilizing SPSS Statistics software v19.0 (http://www.spss.com). All data are expressed as means ± standard deviation unless otherwise indicated. Statistically significant values were defined as p < 0.05.

3 | RESULTS AND DISCUSSION

Transient exposure of isolated oesophageal segments to alkali injury (Figure 1A) induced liquefactive necrosis resulting in corrosive oesophagitis characterized by epithelial sloughing, disruption of the muscularis mucosa and muscularis externa layers, as well as mucosal infiltration of CD68+ macrophages and myeloperoxidase + neutrophils (Figure 1B). Rat survival rate by 2 days postinjury was 76% (52/68) and without surgical intervention 100% mortality (5/5) was observed by 1 week following NaOH exposure with early euthanasia required due to hypersalivation, weight loss, or stridor; symptoms indicative of oesophageal dysphagia. µ-CT analysis of surviving animals 2 days postinjury revealed a significant 91% mean reduction in the luminal cross-sectional area of the injured sites in respect to T7 reference levels (Figure 1C, D). Published reports of NaOH ingestion in various rat models have observed a positive correlation between submucosal collagen deposition and a reduction in luminal oesophageal cross-sectional area consistent with oesophageal stricture formation from excessive extracellular matrix formation during wound healing (Okata et al., 2011; Senturk, Sen, Pabuccu, Unsal, & Meteoglu, 2010). However, this trend was not observed in our system potentially due to the difference in postinjury endpoints of 2 days in comparison to 1 month used in previous studies (Okata et al., 2011; Senturk et al., 2010). Our data imply that acute inflammatory responses following NaOH exposure are the likely mediators of oesophageal stenosis detected in the current setting. Oesophageal reconstruction was performed 3 days following caustic burn injury due to the 100% mortality rate observed in animals by 1 week wherein no surgical intervention was performed.

Prior to scheduled euthanasia, the survival rate of injured rats repaired with BLSF scaffolds was 81% (22/27) with animal death occurring in the initial 5 days postimplantation due to oesophageal obstruction from fur ingestion (4/5) or peritonitis due to a putative leak at the anastomotic perimeter (1/5). In parallel, nondiseased animals subjected to onlay esophagoplasty demonstrated a 92% survival rate.
Constructive tissue remodelling and host tissue responses were temporally evaluated in damaged oesophagi following BLSF matrix implantation by MTS analysis (Figure 3). At 1 week postoperatively, a fibrovascular scar populated by mononuclear inflammatory cells as well as myofibroblasts was evident at graft sites and lined by a stratified squamous, keratinized epithelium. The de novo oesophageal wall contained residual scaffold fragments with putative sites of macrophage phagocytosis located around the perimeters. Invasion of host skeletal muscle fibres and smooth muscle bundles were localized at the peripheral boundaries of the neotissues. At 1 months postimplantation, an ECM-rich lamina propria had developed and both the de novo muscularis mucosa and muscularis externa had further integrated into the graft region. In addition, organization of the muscularis externa into circular and outer longitudinal skeletal muscle layers was apparent at the edges of the neotissues; however, fibrosis still persisted toward internal areas. The de novo muscularis externa at 2 months following repair of injured sites demonstrated a qualitative increase in skeletal muscle density within the central regions of the graft site in comparison to early timepoints. Areas of fibrosis had also diminished at this stage of regeneration and no chronic inflammatory reactions were noted. The structural architecture of neotissues generated in the setting of caustic damage was qualitatively similar to the nondiseased repair group at 2 months postoperatively. However, maturation of the muscularis mucosa and muscularis externa compartments in both these cohorts was notably underdeveloped in respect to nonsurgical controls. Finally, the temporal stages of wound healing encountered during repair of caustic injury sites with BLSF scaffolds were found to mimic the regenerative responses previously observed in our nondiseased model of oesophagoplasty (Algarrahi et al., 2015).
IHC (Figure 4A) and parallel histomorphometric (Figure 4B) evaluations were executed on experimental groups to characterize further the phases of regeneration during repair of damaged regions and compare the degree of tissue maturation achieved between diseased and nondiseased counterparts. Pan-CK+ epithelia were first observed spanning reconstructed injury sites at 1 week following injury. (Figure 2a) Body weight evaluations of nondiseased (BLSF-ND) and diseased (BLSF-ST) animals over the course of the 2 months of scaffold implantation period. Means ± standard deviation per data point. (b) Neotissues present within the original graft sites at 2 months postoperatively. Proximal/distal and lateral marking sutures are respectively designated by red and black arrows. (c) Representative three-dimensional μ-CT images of oesophagi 2 days after NaOH exposure (ST) and in BLSF-ND and BLSF-ST groups at 2 months postoperatively detailed in (b) following contrast agent (red) gavage. Repaired sites in nondiseased and diseased groups are coded in yellow while proximal/distal marking sutures are colored in blue. E = oesophagus. S = stomach. (d) Quantification of luminal oesophageal cross-sectional areas in yellow regions described in (c) and in nonsurgical controls (NSC). (*) = p < 0.05 in comparison to all other groups [Colour figure can be viewed at wileyonlinelibrary.com] (Figure 3) MTS analyses of constructive remodelling at implant sites in experimental cohorts. (first row) Photomicrographs of gross oesophageal cross-sections stained with MTS from nondiseased (BLSF-ND) and diseased (BLSF-ST) groups containing original graft site (bracketed) as well as nonsurgical controls (NSC). Scale bars = 1.25 mm. (second row) Magnification of global regenerative areas (RA) bracketed in first row or the native tissue in NSC. Scale bars = 400 μm. (third row) Magnified boxed green area from second row profiling muscularis externa development. (fourth row) Magnified boxed red area from second row detailing epithelial formation. Scale bars for third and fourth rows = 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]
matrix grafting. By 2 months postoperatively, no significant differences in the extent of pan-CK+ epithelia were noted between implant groups or nonsurgical controls. In addition, distinct epithelial subpopulations were present in all experimental groups and consisted of CK14+ basal cells, polygonal CK4+ suprabasal cells, and flattened FG+ superficial cells. Regenerated vascular networks containing vessels lined with CD31+ endothelial cells were found in both nondiseased and diseased graft sites at all examined timepoints. The mean vessel density was significantly higher in consolidated tissues from all groups in respect to nonsurgical control levels indicating an ongoing stage of tissue remodelling (Zawicki, Jain, Schmid-Schoenbein, & Chien, 1981; Jain, 2003). Analysis of de novo innervation revealed synaptic transmission areas marked by SYP+ boutons in all neotissues. There was no significant difference between the densities of SYP+ boutons in implant

FIGURE 4 Immunohistochemical and histomorphometric assessments of neotissues and controls. (a) Photomicrographs of protein expression patterns in repaired, nondiseased (BLSF-ND) and diseased (BLSFS) graft sites as well as nonsurgical controls (NSC) of epithelia markers, pan-CK, CK14, CK4, FG; endothelial and innervation markers, CD31 and SYP in mucosa; and contractile muscle markers, α-SMA and MYH in muscularis mucosa and muscularis externa, respectively. Arrowheads indicate SYP+ boutons. For all panels, respective marker expression is displayed in red (Alexa Fluor 594 labelling), green (Alexa Fluor 488 labelling), or white (Alexa Fluor 647 labelling) and blue denotes DAPI nuclear counterstain. For all panels, scale bars = 200 μm. (b) Histomorphometric analyses of marker expression in regenerated tissues as well as NSC. (*) = p < 0.05 in comparison to respective NSC. (#) = p < 0.05 in comparison to all other groups [Colour figure can be viewed at wileyonlinelibrary.com]
groups at 2 months postoperatively compared to nonsurgical control levels. Characterization of the regenerated muscularis externa in neotissues demonstrated the extent of MYH+ skeletal muscle in diseased and nondiseased cohorts had respectively achieved 67% and 76% of control values by 2 months of scaffold implantation. In contrast to other tissue components, animals subjected to caustic injury displayed a reduced capacity to support regeneration of the muscularis mucosa in respect to noninjured subjects. The density of α-SMA+ smooth muscle bundles in this compartment was significantly lower in the diseased setting reflecting 33% of nonsurgical control levels by 2 months postoperatively, while nondiseased counterparts were capable of supporting 67%. Taken together, these data demonstrate the ability of BLSF grafts to promote the formation of innervated, vascularized oesophageal tissues at sites of caustic injury; however, the propensity for muscularis mucosal regeneration is muted in comparison to nondiseased microenvironments.

Peristaltic waves generated from radially symmetrical contraction and relaxation of oesophageal circular muscle is an essential mechanism for propagation of food bolus through the digestive tract. Contractile behaviours in repaired conduits from each implant group as well as nonsurgical controls and injured segments prior to scaffold implantation were assessed in ex vivo organ bath studies following stimulation with EFS (Figure 5A) and KCl (Figure 5B). In addition, relaxation properties of experimental groups precontracted with carbachol were analysed following isoproterenol treatment (Figure 5C). Alkali injury to oesophageal tissues significantly decreased both contractile and relaxation responses to all stimuli tested in comparison to nonsurgical controls. These results are consistent with our histological findings demonstrating extensive caustic damage to oesophageal muscular components following transient exposure to NaOH. Following 2 months of BLSF matrix grafting, both repaired nondiseased and diseased conduits demonstrated the ability to contract in response to EFS and KCl stimulation to similar extents. The magnitude of KCl and EFS-induced force generation in these groups was substantially higher than in injured controls, but less than nonsurgical counterparts, suggesting partial recovery of contractile machinery elicited by membrane depolarization as well as neuronal input, respectively. Parallel analysis of relaxation patterns in experimental groups demonstrated similar trends as observed for contractile responses.

4 | CONCLUSIONS

The data presented in this study demonstrate the ability of BLSF grafts to promote functional repair of caustic injury sites in a rat model of onlay oesohagoplasty. Reconstruction of damaged tissues with BLSF scaffolds restored luminal oesophageal calibre, enabled solid food consumption, and promoted the formation of innervated, vascularized epithelial and muscular wall components. In addition, injured oesophageal conduits repaired with BLSF grafts displayed contractile and relaxation properties. In comparison to nondiseased settings, BLSF matrices displayed similar regenerative capacities during healing of damaged regions except for a reduced ability to reconstitute the muscularis mucosa to nonsurgical control levels. In summary, these results provide preclinical validation for the potential of BLSF grafts to serve as off-the-shelf scaffolds for reconstruction of oesophageal tissues following caustic injury.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
JRM and KA designed and conceptualized this study. Acquisition of data was carried out by KA, DF, VC, XY, AS, SA, FMS and MPS. Analysis and interpretation of data was done by JRM, KA, DF, VC, AS and MPS. Manuscript was drafted by JRM, KA, VC and, MPS, while VC, MPS, CRE and JRM performed critical revision of the manuscript for important intellectual content. Statistical analysis was done by KA, VC and MPS. Funding was obtained by JRM and CRE. The entire study was supervised by JRM.

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