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The use of bi-layer silk fibroin scaffolds and small intestinal submucosa matrices to support bladder tissue regeneration in a rat model of spinal cord injury

Yeun Goo Chung [a, 1], Khalid Algarrahi [a, 1], Debra Franck [a], Duong D. Tu [a, b], Rosalyn M. Adam [a, b], David L. Kaplan [c], Carlos R. Estrada Jr. [a, b, **], Joshua R. Mauney [a, b, *]

[a] Urological Diseases Research Center, Boston Children's Hospital, Boston, MA 02115, USA
[b] Department of Surgery, Harvard Medical School, Boston, MA 02115, USA
[c] Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA

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ABSTRACT

Adverse side-effects associated with enterocystoplasty for neurogenic bladder reconstruction have spawned the need for the development of alternative graft substitutes. Bi-layer silk fibroin (SF) scaffolds and small intestinal submucosa (SIS) matrices were investigated for their ability to support bladder tissue regeneration and function in a rat model of spinal cord injury (SCI). Bladder augmentation was performed with each scaffold configuration in SCI animals for 10 wk of implantation and compared to non-augmented control groups (normal and SCI alone). Animals subjected to SCI alone exhibited a 72% survival rate (13/18) while SCI rats receiving SIS and bi-layer SF scaffolds displayed respective survival rates of 83% (10/12) and 75% (9/12) over the course of the study period. Histological (Masson's trichrome analysis) and immunohistochemical (IHC) evaluations demonstrated both implant groups supported *de novo* formation of smooth muscle layers with contractile protein expression [α-smooth muscle actin (α-SMA) and SM22α] as well as maturation of multi-layer urothelia expressing cytokeratin (CK) and uroplakin 3A proteins. Histomorphometric analysis revealed bi-layer SF and SIS scaffolds respectively reconstituted 64% and 56% of the level of α-SMA+ smooth muscle bundles present in SCI-alone controls, while similar degrees of CK+ urothelium across all experimental groups were detected. Parallel evaluations showed similar degrees of vascular area and synaptophysin+ boutons in all regenerated tissues compared to SCI-alone controls. In addition, improvements in certain urodynamic parameters in SCI animals, such as decreased peak intravesical pressure, following implantation with both matrix configurations were also observed. The data presented in this study detail the ability of acellular SIS and bi-layer SF scaffolds to support formation of innervated, vascularized smooth muscle and urothelial tissues in a neurogenic bladder model.

1. Introduction

Spinal cord injury (SCI) and congenital neural tube defects such as myelomeningocele frequently disrupt voluntary control of micturition and lead to neurogenic bladder dysfunction [1]. In patients with a neurogenic bladder, disruption of normal neural pathways induces detrusor sphincter dyssynergia (DSD) causing urinary retention, a functional bladder outlet obstruction, and bladder overdistension [2,3]. This process results in extensive fibroproliferative tissue remodeling of the detrusor muscle which can ultimately lead to diminished bladder capacity and poor compliance [4,5]. Urologic complications secondary to spinal cord disorders including urinary incontinence, recurrent urinary tract infections and renal insufficiency or failure severely impact patient quality of life and contribute substantially to the clinical costs of disease management [6,7]. Standard treatment of the neurogenic bladder typically involves clean intermittent catheterization coupled with anti-cholinergic agents or botulinum toxin in order to

* Corresponding author. Boston Children's Hospital, Department of Urology, John F. Enders Research Laboratories, 300 Longwood Ave., Rm. 1009, Boston, MA 02115, USA. Tel.: +1 617 919 2521; fax: +1 617 730 0248.
** Corresponding author. Boston Children's Hospital, Department of Urology, 300 Longwood Ave., Hunnewell 3, Boston, MA 02115, USA. Tel.: +1 617 355 3338; fax: +1 617 730 0474.
E-mail addresses: carlos.estrada@childrens.harvard.edu (C.R. Estrada), joshua.mauney@childrens.harvard.edu (J.R. Mauney).
[1] Equal contribution.

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mitigate the risk of renal damage from high pressure voiding and detrusor overactivity [8,9]. In cases where conservative therapies have failed, augmentation cystoplasty with autologous gastrointestinal segments represents the primary option for increasing bladder capacity and compliance in order to preserve upper urinary tract function [10]. However, this strategy is associated with substantial complications including metabolic abnormalities, chronic urinary tract infections, bowel dysfunction, and secondary malignancies [11,12].

Tissue engineering approaches utilizing biodegradable scaffolds either alone or seeded with primary cell sources have been explored as alternatives to enterocystoplasty for management of neurogenic bladder dysfunction in both animal models [13–15] and clinical trials [16,17]. In particular, decellularized collagen-based bladder acellular matrix (BAM) has been previously utilized as an acellular graft for bladder augmentation in a rat model of spinal cord injury [13–15]. In these reports, BAM was capable of promoting regeneration of innervated, vascularized smooth muscle and urothelial tissues within implantation sites by supporting host tissue integration [13–15]. Improvements in urodynamic parameters such as bladder capacity and compliance were also evident in SCI animals following BAM grafting [14,15]. Overall, the ability of BAM to encourage functional tissue regeneration within the setting of a neurogenic bladder was found to be comparable to the results observed in healthy animals [14]. Nonetheless, substantial adverse effects including atrophy of grafted BAM, pyuria, implant contracture, urinary tract infections, and urinary calculi were still observed in the augmented SCI model [13,14] raising concerns over the translational potential of this biomaterial for clinical deployment.

Short-term clinical trials reported by Atala and colleagues first demonstrated the ability of a collagen-coated poly-glycolic acid mesh, seeded with patient-derived smooth muscle and urothelial cells expanded *ex vivo*, to mediate *de novo* bladder tissue formation in children with myelomeningocele [16]. However, phase II studies of this technology at 3 years post-implantation failed to show significant improvements in bladder capacity or compliance within the neurogenic bladder population [17]. In addition, the level of serious adverse events including bowel obstruction and bladder rupture encountered with this approach were reported to surpass an acceptable safety standard [17]. Therefore, there exists a substantial need for the development of novel methods for bladder reconstruction in patients with spinal cord defects.

We hypothesized that an optimal strategy for augmentation cystoplasty of the neurogenic bladder would consist of an "off-the-shelf" acellular graft with the structural, mechanical, and degradation properties sufficient to support initial defect stabilization while allowing for gradual remodeling, host tissue ingrowth, and subsequent tissue regeneration without adverse immunogenic reactions. Bi-layer silk fibroin (SF) scaffolds derived from *Bombyx mori* silkworm cocoons as well as porcine small intestinal submucosa have been previously shown to promote defect consolidation and mediate functional voiding in non-diseased animal models of bladder augmentation [18–24]. These matrices, therefore, represent potential candidates for neurogenic bladder repair; however their performance in the setting of neuropathogenic disease is currently unknown. In the present study, we investigated the efficacy of these scaffolds to support tissue regeneration and bladder function in a rat model of SCI.

2. Materials and methods

2.1. Biomaterials

Aqueous SF solutions were prepared from *B. mori* silkworm cocoons using published procedures [25] and utilized to construct a bi-layer SF matrix using methods previously described [24]. Briefly, an SF solution (8% wt/vol) was poured into a rectangular casting vessel and dried in a laminar flow hood at room temperature for 48 h to achieve formation of an SF film. A 6% wt/vol SF solution was then mixed with sieved granular NaCl (500–600 μM, average crystal size) in a ratio of 2 g NaCl per ml of SF solution and layered on to the surface of the SF film. The resultant solution was allowed to cast and fuse to the SF film for 48 h at 37 °C and NaCl was subsequently removed by washing the scaffold for 72 h in distilled water with regular volume changes. The morphology of the bi-layer SF scaffold has been previously reported [24]. Briefly, the solvent-cast/NaCl-leached layer comprised the bulk of the total matrix thickness (2 mm) and resembled a foam configuration with large pores (pore size, ~400 μm) interconnected by a network of smaller pores dispersed along their periphery. This compartment was buttressed on the external face with a homogenous, non-porous SF layer (200 μm thick) generated by film annealment during casting. Before implantation, bi-layer SF scaffolds were sterilized in 70% ethanol and rinsed in phosphate buffered saline (PBS) overnight. SIS grafts (Cook, Bloomington, IN) were evaluated in parallel. Tensile properties of both scaffold configurations have been previously reported [24].

2.2. SCI model and bladder augmentation

Forty-two female Sprague–Dawley rats (6 wk of age, Charles River Laboratories, Wilmington, MA) were subjected to complete extradural spinal cord transection at the 8th thoracic level (T8) to elicit a previously described reflex bladder phenotype with overactivity [26]. This model has been reported to produce spinal cord lesions containing glial scarring as well as neuronal damage, consisting of neuronal swelling and chromatin dispersion with focal chromatolysis [26]. Under general anesthesia induced by isoflurane inhalation, a dorsal midline incision was made through the superficial and deep muscle layers over the thoracic spinal cord. Posterior laminectomy was executed and a dorsal transection across the entire width of the spinal cord at T8 was performed with a scalpel blade. Excision of 2–3 mm of spinal cord tissue was carried out to ensure complete transection and the subsequent gap was filled with absorbable gelatin sponge (Ethicon™). Muscle and skin layers were subsequently closed with absorbable sutures. Post-operative pain was managed with meloxicam (1 mg/kg, subcutaneously) analgesia. During the period of spinal shock, which lasted from 1 to 3 wk following creation of SCI, bladders of rats were emptied twice daily by manual compression with care taken to avoid unintentional bladder rupture.

Following 6 wk of SCI, 24 animals were divided into 2 groups of 12 and subjected to augmentation cystoplasty with either SIS (Group 1, SCI–SIS) or bi-layer SF scaffolds (Group 2, SCI–SF) for 10 wk of implantation as previously described [24,27]. The 14 remaining SCI animals were maintained similarly in parallel as longitudinal controls without matrix grafting (Group 3, SCI-control). For bladder augmentation studies, animals were anesthetized using isoflurane inhalation and then shaved to expose the surgical site. A low midline laparotomy incision was then made and the underlying tissue (rectus muscle and peritoneum) was dissected free to expose the bladder. Four traction sutures (7-0 polypropylene) were placed in a square configuration. A longitudinal cystotomy incision was then made in the bladder dome in the middle of these traction sutures using fine scissors to create a bladder defect (Fig. 1A). A circular piece of biomaterial (~10 mm in diameter) was then anastamosed to this site using 7-0 vicryl continuous suture (Fig. 1B). Non-absorbable 7-0 polypropylene sutures were placed at the edges of the implantation area for identification of graft borders. A watertight seal was confirmed by filling the bladder with sterile saline via instillation through a 30 gauge hypodermic needle. Skin incisions were subsequently closed with running sutures. At 10 wk post-augmentation, animals were harvested for endpoint evaluations described below. In addition, 11 normal rats not receiving bladder implants (Group 4, NS-Control) were analyzed in parallel as a positive control cohort. All animal studies were approved by the Boston Children's Hospital Animal Care and Use Committee prior to experimentation.

2.3. Cystometric analyses

Bladder urodynamics were evaluated in all experimental groups using conscious unrestrained cystometry previously described [24,26,27]. A suprapubic (SP) catheter was surgically inserted into the bladder 1–3 d prior to cystometry. Under isoflurane anesthesia, a laparotomy was created using a ventral lower midline incision. A flared tip SP catheter (polyethylene-50; Intramedic, Sparks, MD) was inserted into the bladder, secured with 6-0 prolene purse-string suture, and tunneled through the subcutaneous area to a dorsal midline skin incision between the scapulae. An access port was connected to the outlet of the SP catheter on the dorsal aspect with a luer-lock inter-link system (Injection site; Baxter, Deerfield, IL).

During cystometry, the SP catheter was attached to a physiological pressure transducer (model MLT844, ADInstruments, Colorado Springs, CO) to allow measurement of intravesical pressure, while the bladder was continuously infused with sterile PBS at 100 μl/min. Pressure readings were digitized using a PowerLab data acquisition system and analyzed using LabChart Pro software (ADInstruments, Colorado Springs, CO). Post void residual volumes were measured by aspirating the SP catheter at the conclusion of cystometry. After establishment of a regular voiding pattern, multiple other variables were extrapolated from the cystometric tracings, such as compliance, spontaneous non-voiding contractions (SNVC), resting pressure,

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