Received: 24 September 2019 | Revised: 8 January 2020 | Accepted: 19 January 2020

DOI: 10.1002/jbm.b.34576

Society For Biomaterials

WILEY

ORIGINAL RESEARCH REPORT

Evaluation of 1-mm-diameter endothelialized dense collagen tubes in vascular microsurgery

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Funding information
National Institute of Biomedical Imaging and Bioengineering, Grant/Award Numbers: EB006359, EB018851

Abstract

Although much progress has been made in engineering vascular grafts for large- and small-diameter arterial repair or bypass, the extension of these results to the microsurgical size scale has been challenging. Here, we evaluated the use of dense collagen tubes (outer diameter 1 mm, inner diameter 0.5 mm) for vascular microsurgery as interpositional grafts to the femoral artery of Lewis rats. These tubes were formed by dehydrating tubular collagen gels around a mandrel, crosslinking them with genipin, seeding with syngeneic endothelial cells, and culturing before implantation by suture anastomosis. The retention of a confluent endothelial lining inside the tubes after mock surgical handling depended strongly on the crosslinker concentration and culture time. Optimized preparation conditions enabled retention of endothelium after mock surgical handling in ~80% of tubes and maintenance of patency 7 days after implantation in ~40% of grafts. Histological analysis showed the development of granulation tissue and the presence of CD31-positive structures on the inner and outer surfaces of implants. This study provides a proof-of-principle demonstration that endothelialized dense collagen tubes can remain patent for up to 7 days after vascular microsurgery, and points to the importance of mild scaffold crosslinking for maintaining firm endothelial adhesion.

KEYWORDS

endothelial cells, Lewis rat, microsurgery, vascular tissue engineering

1 | INTRODUCTION

Tubular biomaterials that can conduct blood in vivo range from the glass and metal tubes developed by Carrel in the early 1900s to the synthetic polyester and fluoropolymer meshes currently used for aortic repair in humans. These tubes typically have outer diameters of 1 cm or more. For outer diameters in the range of 3–6 mm (so-called "small-diameter" vascular grafts suitable for coronary or peripheral vascular bypass), the development of appropriate materials has been more challenging. The smaller diameters magnify the deleterious effects of thrombus and/or graft intimal hyperplasia. Nevertheless, many candidate grafts—based mainly on natural biopolymers such as fibrin and Type I collagen—have been developed for small-diameter applications (Isenberg, Williams, & Tranquillo, 2006; L'Heureux et al., 2007; Seifu, Purnama, Mequanint, & Mantovani, 2013).

Extension of these results to even finer diameters of 1 mm or smaller has not been as well-explored or successful (Harris & Seikaly, 2002). Grafts at this scale are envisioned for applications in vascular microsurgery (e.g., to bridge vascular stumps in the extremities or in the transfer of free flaps), and they are implanted with specialized microsurgical techniques. It is important to recognize that the design parameters that govern engineered vascular grafts at diameters of 3 mm and higher are different from those at the microsurgical scale. At the larger scale, compliance mismatch between the artery and graft is believed to be a central cause of the intimal hyperplasia that can arise over months to years (Lemson, Tordoir, Daemen, & Kitslaar, 2000). Since these grafts are intended to function for the lifespan of

the recipient, great pains are taken to ensure that the graft has similar mechanical properties as those of an artery of similar size. In contrast, microsurgical grafts only have to remain patent long enough for collateral circulation to naturally develop between the proximal and distal tissues, usually 1–2 weeks (Black, Chait, O'Brien, Sykes, & Sharzer, 1978). Moreover, the diameters are so small at the microsurgical scale that any thrombus rapidly grows to occlude the entire flow area, and some strategy to maintain patency (e.g., endothelialization) appears to be required. Microsurgical implants of unseeded synthetic polymer tubes have shown poor patency rates (Harris & Seikaly, 2002; Lanzetta, 1998).

Techniques that have been investigated for engineering scaffolds suitable for microsurgical vascular grafts with inner diameters (ID) ranging from 0.6 to 1.3 mm include rolling of collagen/elastin sheets (Kumar et al., 2013), dip-coating of a cylindrical mandrel with polyvinyl alcohol (Cutiongco et al., 2016), electrospinning of resorbable polymers (Gao et al., 2016; Kuwabara et al., 2012; Kuwabara et al., 2012), decellularization of small arteries (Yamanaka, Yamaoka, Mahara, Morimoto, & Suzuki, 2018), and using a foreign body response to generate tubes of connective tissue (Ishii et al., 2016; Ishii et al., 2018). While these tubular scaffolds showed promising patency in vivo, they lacked a preformed endothelium. To our knowledge, no study has demonstrated that a collagen-based, endothelialized vascular graft with an outer diameter (OD) of 1 mm can be successfully used in vascular microsurgery.

Recently, we have developed collagen tubes of OD 1 mm that possess mechanical properties comparable to those of rat femoral arteries (Li, Xu, Nicolescu, Marinelli, & Tien, 2017). These scaffolds were inspired by the previous studies of Brown and Nazhat, who showed how densification of collagen gels by plastic compression could greatly increase the mechanical and suture retention strengths of collagen (Abou Neel, Cheema, Knowles, Brown, & Nazhat, 2006; Brown, Wiseman, Chuo, Cheema, & Nazhat, 2005; Ghezzi, Marelli, Muja, & Nazhat, 2012). Our previous study relied on the drying of cylindrical collagen gels into tubes and subsequent crosslinking of the tubes to enhance their mechanical properties. Human umbilical vein endothelial cells (HUVECs) reached confluence on these tubes within 2 days after seeding and remained viable for an additional 5 days. In addition, unseeded tubes withstood arterial flow and pressure when implanted as interpositional grafts in the rat femoral circulation, without the need for mechanical support from a synthetic sleeve. As expected, unseeded microsurgical grafts occluded within ~20 min, most likely from a lack of endothelium to protect the graft from platelet adhesion and activation.

The current study examines the behavior of endothelialized collagen tubes as microsurgical grafts in an immunocompetent animal (rat). We report the short-term (~20 min) and long-term (up to 1 week) behavior of these tubes in the rat femoral circulation. Surprisingly, we found that endothelial stability under surgical handling required less scaffold crosslinking. When optimally crosslinked and cultured, these endothelialized collagen tubes could remain patent for one week after implantation without adjuvant anticoagulant treatment. This study thus provides a proof-of-principle demonstration that such collagen-based tubes can be used successfully in vascular microsurgery.

2 | METHODS

2.1 | Cell culture and characterization

Lewis rat aortic endothelial cells (RAOECs; Cell Biologics) were grown on gelatin-coated tissue culture dishes in a proprietary endothelial cell medium (Cell Biologics). Cells were cultured under 5% CO_2 at 37°C and passaged at a ratio of 1:4 using 0.005% trypsin in phosphate-buffered saline (PBS). Cells were discarded after passage 8.

RAOECs were assayed for uptake of acetylated low-density lipoprotein (AcLDL) uptake to confirm their endothelial nature. At confluence, cells were incubated for four hours at 37°C in culture medium that was supplemented with 5 μg/ml DiI-labeled AcLDL (Alfa Aesar) and rinsed with fresh medium three times. Uptake of AcLDL was visualized by epifluorescence imaging using a ×10/0.3 Plan-Neofluar objective on a Zeiss Axiovert 200 M microscope at 1,040 × 1,388 camera resolution, and was routinely positive in >95% of RAOECs.

2.2 | Formation of endothelialized dense collagen tubes

Dense collagen tubes were made using a previously described method (Figure 1; Li et al., 2017). Briefly, rat tail Type I collagen (Corning) was neutralized to pH 7 and final concentration 6.6 mg/ml and introduced to a cylindrical mold (ID 6 mm) with a 0.5-mm-diameter mandrel in the center. After gelling for two hours at room temperature, the collagen tube was removed from the mold and washed in deionized water for one hour to remove excess salt. It was then placed on a rotisserie and dehydrated overnight with constant rotation, removing ≥99% water.

The dried collagen tubes were then crosslinked with various concentrations of genipin (0.1, 0.5, 1, 5, 10, 15, or 20 mM in PBS; Wako) for 2 hours at room temperature, washed three times for 1 hour each in PBS, and conditioned in medium overnight.

RAOECs were trypsinized and suspended in culture medium at 2 million cells/ml. Medium-conditioned dense collagen tubes were cut into 4- to 5-mm-long segments and submerged in cell suspension. Tubes were gently milked with tweezers to ensure that the cell suspension filled the lumen. While still submerged in cell suspension, the tubes were tumbled for one hour at 37°C. Seeded tubes were washed once with medium, placed in separate wells of a 24-well plate that had been precoated with Pluronic F68 (5 mg/ml in PBS for 1 hour; Sigma) to prevent adhesion, and cultured under static conditions with 0.5 ml medium. Culture medium was replaced every 2 days for up to 10 days.

2.3 | Viability assay

After being cultured for 3 days, seeded tubes were incubated in medium that was supplemented with 10 μg/ml calcein AM and 5 μg/ml Hoechst 33342 (both from Invitrogen) for 30 min. Tubes were then cut open lengthwise for fluorescence imaging of live cells and nuclei in the lumen.

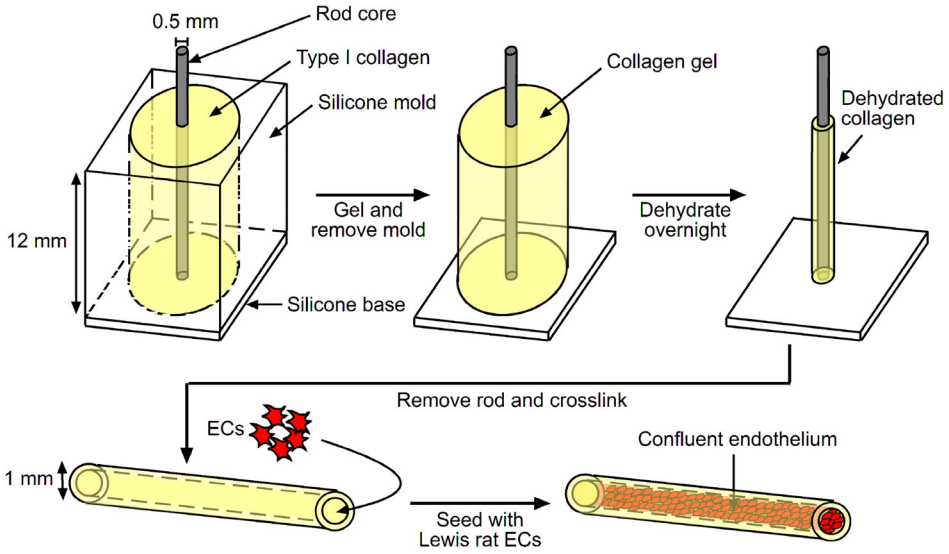

FIGURE 1 Schematic diagram of the formation of endothelialized dense collagen tubes by dehydration of a tubular collagen gel, crosslinking with genipin, and seeding of the lumen with endothelial cells

2.4 | Characterization of endothelial stability in vitro

After being cultured for three or 10 days, endothelialized collagen tubes were gently squeezed with #5 tweezers (Fine Science Tools) twenty times to mimic surgical handling. Squeezed tubes were gently flushed once in PBS to remove any detached endothelial cells. Tubes were fixed in formalin (Fisher) for 30 min and washed three times for 10 min each in PBS. Nuclei were stained with 5 μg/ml Hoechst 33342 for 10 min. Tubes were then cut open lengthwise for fluorescence imaging of nuclei. Tubes that possessed intact endothelium throughout the lumen (not counting slight damage to the edges of the tube from handling with tweezers) were considered "undamaged."

2.5 | Measurement of mechanical properties

Mechanical properties of collagen tubes were determined using the same procedures as described previously (Li et al., 2017). Measurements of ultimate tensile stress (UTS) and compliance used tubes that were crosslinked, seeded, and cultured for 10 days. For UTS measurements, we secured seeded tubes with flat metal clamps at both ends, hooked one clamp to a force gauge (Jonard), and pulled the other clamp at 1 mm/s until the collagen tube fractured. The measured force was divided by the initial cross-sectional area of the collagen wall to calculate UTS. For compliance measurements, we cinched seeded tubes onto a 25-gauge needle at one end and closed the other end with a 6–0 Prolene suture (Ethicon). The needle was connected to pressurized nitrogen gas. The pressure was increased from 0 to 380 mmHg in three increments, and the compliance was calculated by linear regression between pressure and percentage change in outer diameter. Burst pressures were measured similarly, except the tubes were unseeded; values were obtained by gradually increasing the pressure of the system until the sample failed.

All mechanical properties were measured at room temperature.

2.6 | Implantation of seeded dense collagen tubes in vivo

All surgical procedures were performed in accordance with institutional guidelines and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Twenty-two tubes were implanted, with one tube per animal. For each implantation, a female Lewis rat (200–250 g; Charles River) was anesthetized with 4% isoflurane and maintained at 1.5% isoflurane for the duration of the surgery (~4 hr). Buprenorphine (0.1 mg/kg) was administered 30 min before incision. The lower abdomen was opened, and the left femoral artery and vein were liberated. Blood flow in the femoral artery was temporarily interrupted with a microsurgical clamp, and a 2-mm-long segment of femoral artery was excised, leaving a 1-mm-long artery stump at either end. The artery stumps were irrigated with 100 U/ml heparin (Sigma) and trimmed of adventitia. A seeded dense collagen tube that had been maintained at 37°C was anastomosed end-to-end to the femoral artery with eight sterile 10–0 sutures (ARO Surgical) at both ends using an interrupted stitching technique, as previously described (Li et al., 2017). After all sixteen sutures were completed, the microvascular clamp was released to restore blood flow. Acute patency of the implanted collagen tubes were monitored for up to 20 min by performing the "milking test" described by Acland (Acland, 1972). If the implanted tube remained patent with clear pulsation for up to 20 min after reestablishing blood flow, then the wound was closed with surgical clips (Braintree Scientific); if not, the rat was euthanized by CO_2 under anesthesia, followed by bilateral thoracotomy. After wound closure, another 0.1 mg/kg of buprenorphine was administered, and the rat was removed from anesthesia after an additional 30 min. Rats with patent tubes were kept for up to 7 days after surgery, with 0.05 mg/kg buprenorphine administration twice daily for the first 2 days and as needed thereafter. On up to the 7th day, the surgical site was reopened and the patency of the implanted tube was confirmed again with the Acland test. The tube was then excised with 1- to

2-mm-long segments of femoral artery on both ends and fixed with formalin (10%, neutral-buffered) for further characterization. The rat was then euthanized with CO_2 under anesthesia, followed by bilateral thoracotomy.

In three additional (control) rats, a 5-mm segment of femoral artery was excised and immediately replanted in its original position with eight sutures on each end. Wounds were closed and rats were maintained for 7 days. Implanted arteries were then harvested and fixed with formalin for characterization.

In total, implants of endothelialized tubes or vessels were performed on 25 animals. Explantation time was chosen to be up to 7 days, because this time is the minimum required for vascular patency to ensure tissue survival in microsurgery (Black et al., 1978); patency beyond 7 days was not evaluated. No systemic anticoagulation was used.

2.7 | Histological evaluation of implanted tubes

Two tubes that were crosslinked with 20 mM genipin and cultured for 3 days (harvested 3 and 5 days after implantation), three tubes that were crosslinked with 5 mM genipin and cultured for 10 days (all harvested 7 days after implantation), and three replanted femoral arteries (all harvested 7 days after replantation) were histologically evaluated. Tissues were fixed in formalin, embedded in paraffin, and sectioned.

Russel-Movat pentachrome staining kit (American MasterTech) was used in accordance with the manufacturer's instructions for the microscopic structural evaluation of all implanted collagen tubes and sham control anastomoses (i.e., artery replants). Structures that identified as elastic fibers stained dark blue; as collagen, yellow; as fibrinoid, intense red; as muscle, brown-red, and as glycosaminoglycans (GAGs) or mucin, blue-green.

Immunohistochemistry was also performed to evaluate the presence of endothelium in implanted tubes that were crosslinked with 5 mM genipin and in sham control anastomoses. A total of eight random sections per rat were deparaffinized and rehydrated in decreasing alcohol series. Endogenous peroxidase activity was quenched by 1% H_2O_2 and sections were washed in PBS. Protein blocking was performed with horse serum and sections were incubated for 1 hr with mouse anti-rat CD31 monoclonal antibody (clone TLD-3A12; Invitrogen) at a concentration of 1:100 dilution. Mouse IgG (20 μg/ml) as well as omitting the primary or secondary antibodies were used as technical controls. After PBS washes, horseradish peroxidase-conjugated horse anti-mouse IgG (ImmPRESS polymer detection kit; Vector Labs) was applied for 30 min. Sections were washed in PBS and enzyme substrate 3,3′-diaminobenzidine-nickel for 10 min and counterstaining with Nuclear Fast Red (Vector Labs) was applied, followed by dehydration, clearing, and mounting. All conditions were processed simultaneously. Images were captured randomly by a Nikon Eclipse 50i microscope at ×10 and ×4. Histological evaluation was performed by an expert histologist (E.B.-S.).

2.8 | Statistical analysis

All statistical tests were performed with the Prism version 6 software (Graphpad). Chi-squared test for trend was used to assess the effect of scaffold crosslinker concentration on endothelial stability (cultured for either 3 or 10 days after seeding). Fisher's exact test was used to assess the effect of culture time on the stability of endothelium and to compare in vivo patency rates. One-way ANOVA with Dunnett's post-hoc tests was used to compare burst pressures for different crosslinker concentrations. UTS, burst pressure, and compliance were reported as mean ± standard deviation. A value of $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Rat aortic endothelial cells reached confluence in dense collagen tubes

Previously, we seeded dense collagen tubes with HUVECs and showed that these cells reached confluence in 2 days and remained viable for up to 7 days (Li et al., 2017). The use of HUVECs precluded the evaluation of these tubes as grafts in an immunocompetent host without rejection. In the current study, we seeded collagen tubes with aortic endothelial cells from Lewis (inbred) rats, so that the resulting tubes could be implanted without rejection. As found previously for HUVECs, RAOECs adhered and grew well in the collagen tubes. Indeed, RAOECs remained viable and reached confluence by 3 days postseeding (Figure 2a).

3.2 | Unoptimized crosslinking and culturing method led to poor patency rate in vivo

Initial implants of endothelial tubes used scaffolds that were crosslinked with 20 mM genipin and cultured for 3 days after seeding with RAOECs. This procedure was based on our previous preparation conditions for HUVEC-seeded tubes (Li et al., 2017). Although this choice of crosslinker concentration and culture time yielded tubes with confluent endothelium (Figure 2a) and with sufficient mechanical strength to withstand suture anastomosis and arterial flow (Figure 2b), it resulted in surprisingly poor patency rate after anastomosis. In fact, only 2 out of 11 implanted tubes remained patent at 20 min after reestablishing blood flow. Of these two tubes, only one remained grossly patent 5 days after implantation. Moreover, histological staining of this tube showed that thrombus had formed inside the lumen (Figure 2c). The other tube was no longer patent 3 days after surgery, and its lumen was completely occluded by thrombus (Figure 2d). That is, none of the eleven implants could maintain a thrombus-free lumen. These in vivo findings suggested that, although an endothelium was well-formed on tubes in vitro, it was lost and/or thrombogenic after implantation in vivo.

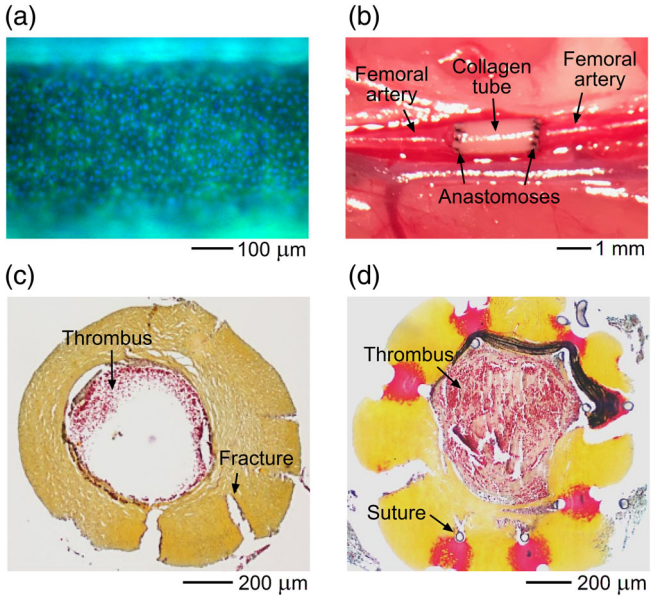

FIGURE 2 Implantation of seeded dense collagen tubes (crosslinked with 20 mM genipin, cultured for 3 days). (a) Representative *en face* view of live (green) and nuclear (blue) stain of a seeded collagen tube. (b) Intraoperative view of an endothelialized tube as interpositional graft on Day 0. (c) Histological stain of a partially clotted tube explanted on Day 5. (d) Histological stain of a completely clotted tube explanted on Day 3

3.3 | Endothelial stability depends on crosslinking and culturing conditions in vitro

Histological sections of explanted tubes that had been crosslinked with 20 mM genipin and cultured for 3 days showed that these tubes possessed cracks in their walls (Figure 2c,d). These cracks most likely resulted from the surgical handling required during anastomosis, in which the tubes were repeatedly squeezed or stretched to assist in insertion of suture and tying of stitches. The presence of cracks suggested that the collagen gels might be brittle, and we expected the cracks to be detrimental to firm endothelial cell adhesion. Indeed, when similarly prepared tubes were gently squeezed 20× in vitro to mimic surgical handling and then flushed with PBS, large patches of the lumen area became denuded of endothelium (Figure 3a, image labeled "20 mM"). This result could potentially explain the poor patency rates of these tubes in vivo, as bare collagen is extremely thrombogenic.

We investigated whether lowering the stiffness of the collagen gel might improve the mechanical stability of the endothelium. In addition, we examined whether culturing endothelial cells for a longer time after seeding would also lead to improved stability. The tubes were crosslinked with genipin at concentrations as low as 0.1 mM, cultured statically for up to 10 days, and then subjected to mock surgical handling. For tubes that were cultured for 3 days, endothelial stability improved as the concentration of genipin was decreased (p = .0055). Nevertheless, patches of denuded surface were apparent after squeezing (Figure 3a).

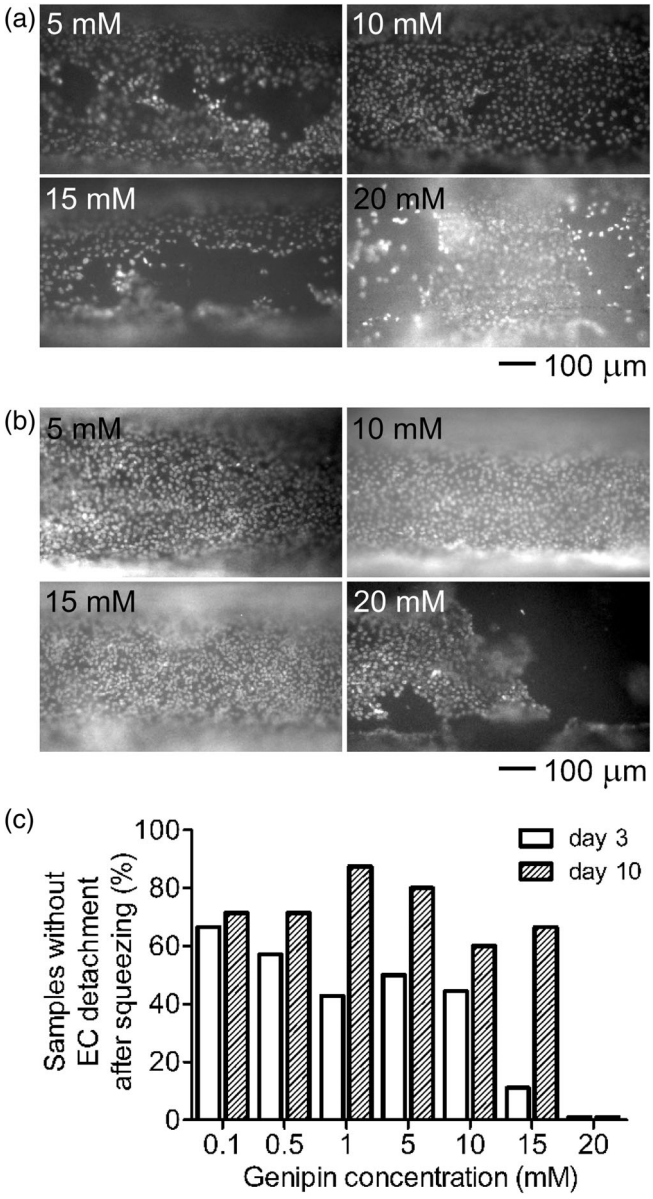

FIGURE 3 In vitro stability of endothelium on collagen tubes with different crosslinking conditions and culture times. (a) Representative *en face* images of tubes that were cultured for 3 days, subjected to mock surgical handling, flushed with PBS, and stained with Hoechst. (b) Representative images for tubes that were cultured for 10 days. Results for tubes crosslinked with 0.1, 0.5, or 1 mM genipin were similar to those shown for tubes crosslinked with 5 mM genipin. (c) Fraction of samples that possessed intact endothelium after mock surgical handling, as a function of crosslinker concentration and culture time

Endothelial stability was further improved by increasing the culturing time. After 10 days of culturing, intact endothelium was present in 70–90% of samples tested, when the crosslinker concentration was 5 mM or less (Figure 3b). Mock surgical handling of tubes that were crosslinked with 20 mM genipin still resulted in damaged endothelium after 10 days of culturing (Figure 3b, image labeled "20 mM"). These results showed that decreasing genipin concentration improved the

stability of endothelium after 10 days of culture (p = .018). Increasing culture period from 3 to 10 days increased endothelial stability (p = .014).

3.4 | Tubes with optimized gelling and seeding conditions possess sufficient mechanical strength for implantation

Our in vitro results showed that crosslinking with lower genipin concentration, as well as culturing for longer times before implantation, would increase the ability of endothelium to resist the mechanical stresses of microsurgery. Reducing the crosslinker concentration, however, could be accompanied by weakening of the tube wall. Thus, we measured the burst pressure of tubes that were crosslinked with various concentrations of genipin. These measurements showed that burst pressure was consistently greater than 1,000 mmHg for genipin concentrations of 5 mM or higher, but decreased precipitously for 1 mM or smaller (p < .0001; Figure 4). Altogether, treatment of tubes with 5 mM genipin and culture for 10 days gave the optimal combination of endothelial stability (favored by low genipin concentrations and long culture times) and burst resistance (favored by high genipin concentrations).

For these optimally prepared tubes, the burst pressure was 1,186 ± 76 mmHg, the UTS was 2.4 ± 0.6 MPa, and compliance was 3.2 ± 0.8%/100 mmHg. These values compare favorably to values for tubes that were crosslinked with 20 mM genipin (1,250 ± 142 mmHg for burst pressure in the current work; 1,313 ± 156 mmHg for burst pressure, 2.1 ± 0.6 MPa for UTS, and 1.7 ± 0.4%/100 mmHg for compliance in previous work (Li et al., 2017)). These comparable values indicated that dense collagen tubes that were crosslinked with 5 mM genipin and cultured for 10 days should retain enough mechanical strength to withstand arterial perfusion, without compromising endothelial stability. Since we have shown previously that UTS and suture retention strength are highly correlated in dense collagen tubes (Li et al., 2017), it is likely that these optimized conditions would also be mechanically sufficient for suture anastomosis.

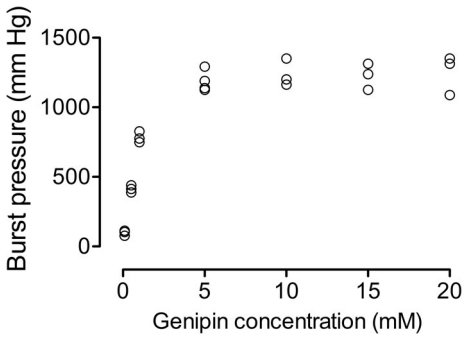

FIGURE 4 Plot of burst pressure of collagen tubes as a function of crosslinker concentration

3.5 | Optimized implants conducted blood flow for up to 7 days in vivo

To test whether the improved mechanical and biological properties of the optimized tubes would lead to better patency in vivo, we implanted tubes that were crosslinked with 5 mM genipin, seeded, and cultured for 10 days. Of the eight implants, three remained patent at 20 min after implantation and reestablishment of femoral circulation in vivo. Importantly, all three tubes maintained stability without rejection, and were patent when the surgical sites were reopened after 7 days. Gross examination showed that a thin layer of vascularized connective tissue had formed on the outer surface of the implanted tubes, the anastomoses, and the femoral vessels, and

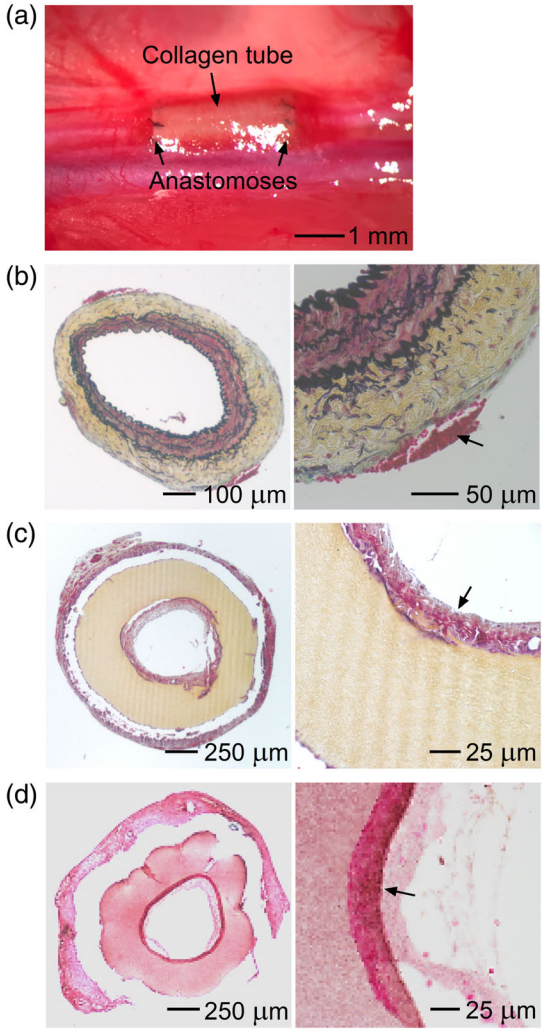

FIGURE 5 Histological characterization of patent endothelialized tubes (crosslinked with 5 mM genipin, cultured for 10 days) 7 days after implantation. (a) Intraoperative view of implanted tube at Day 7 while it was still connected to femoral circulation. (b,c) Pentachrome-stained images of sham replanted artery (b) and implanted tube (c). Arrows indicate granulation tissue. (d) CD31 immunohistochemical stain of implanted tube. CD31 is dark brownish (arrow) and nuclei are pink. Right panels are magnified views of selected areas in the left panels

served to anchor the implant to the surrounding tissue (Figure 5a). In contrast, three implants of tubes that were prepared under sub-optimal conditions (crosslinked with 10 mM genipin and/or cultured for 3 or 5 days) led to immediate thrombosis.

Pentachrome-stained images revealed that the lumens of sham surgery and three of eight implanted tubes (5 mM genipin, 10-day culture) remained patent without thrombosis or infection at 7 days after surgery. Compared to all other crosslinking and culture conditions, the combination of 5 mM genipin and 10-day culture led to an increase in the long-term, thrombus-free patency rate (p = .036). Arteries from sham surgery showed undisturbed intima, media, and adventitia layers (Figure 5b). In these surgical controls, loose reddish granulation tissue occurred at the suture sites as a result of injury (Figure 5b, arrow). All three explanted tubes also showed similar level of granulation tissue formation on the inside and outside surface of the tube (Figure 5c, arrow), as well as on the proximal and distal segments of femoral artery at the anastomosis sites. The granulation tissue that lined the inner surface of the tubes was ~25 μm thick and was consistent with an inflammatory phase of repair with fibrinogen deposition, platelet aggregation, and some neutrophil and macrophage infiltration (Figure 5c). A few fibroblasts also appeared to be present. The outer surface of the implanted tubes exhibited a layer of tissue with similar thickness and histological characteristics as that on the inner surface. Newly formed capillaries were occasionally present in the granulation tissue on the outer surface. The internal volume of the collagen tubes, however, appeared largely cell-free at time of explantation.

Immunohistochemistry for CD31 in implanted tubes (Figure 5d, arrow) showed signal at the inner and outer surfaces. It should be noted that, on the inner surface, CD31 is expressed not only by endothelial cells, but also by platelets and monocytes.

4 | DISCUSSION

In this study, we implanted endothelialized dense collagen tubes (ID 0.5 mm, OD 1 mm) into the rat femoral circulation and demonstrated that ~40% of optimally prepared tubes remained patent and conducted blood flow for 7 days. We tested the endothelial stability in vitro by simulating surgical handling, and found that crosslinking at lower genipin concentration and increasing culture duration after seeding greatly improved endothelial stability. These results build upon and extend our previous work, in which we showed that dense collagen tubes produced by dehydrating and crosslinking native collagen could be endothelialized and possessed sufficient mechanical strength to withstand anastomosis and arterial perfusion (Li et al., 2017).

In the current study, we observed that collagen tubes that were crosslinked with 20 mM genipin and cultured for 3 days after seeding (the same conditions used in the previous study) led to <20% immediate patency after implantation and 0% long-term thrombus-free patency. Subsequent in vitro tests to mimic surgical handling revealed that the endothelium detached after even very gentle squeezing, which explains the observed low patency rate. Cell detachment often occurred in large sheets, rather than as individual cells. Thus, even though endothelial cells spread and grew well on these collagen tubes and withstood the stresses associated with routine cell culture, the adhesion strength was insufficient for the envisioned microsurgical application.

How could a milder crosslinking and longer cell culture period enhance endothelial stability? Since endothelial cells secrete adhesive extracellular matrix proteins (Kramer, Bensch, Davison, & Karasek, 1984; Kramer, Fuh, Bensch, & Karasek, 1985; Kramer, Fuh, & Karasek, 1985), the greater accumulation of such proteins with longer culture could increase the adhesion strength and mechanical robustness of the endothelium. It is less clear why crosslinking at lower genipin concentrations could enhance stability, as endothelial cell spreading, traction forces, and presumably adhesion strength are greater on stiffer substrata (Califano & Reinhart-King, 2010). One clue is that the weakness of the endothelial cell adhesion to collagen tubes was only apparent after repeated mechanical squeezing of seeded tubes. This finding, coupled with the prevalence of large cracks in the tube wall after squeezing of highly crosslinked samples, suggested that the interaction between cracks in the collagen and the overlying cells could be partly responsible for the delamination of endothelium. Alternatively, mechanical mismatch between the endothelium and collagen tube may lead to delamination upon sample deformation, a phenomenon that has been observed during flexing of rigid substrates that are coated with thin films (Mei, Huang, Chung, Stafford, & Yu, 2007).

Tubes that were formed under optimized conditions (crosslinked with 5 mM genipin and cultured for 10 days after seeding) showed relatively high endothelial stability in vitro (~80% intact endothelium after simulated surgical handling). In contrast, the immediate patency rate of these optimized tubes in vivo was lower (~40%). A possible explanation of the discrepancy between in vitro and in vivo results is that during actual surgery, the seeded tubes were exposed to room temperature in a serum-free environment for 1–2 hr, which may adversely affect the endothelial stability. Furthermore, the actual surgical handling was more rigorous compared to the in vitro test, which would cause more damage to the endothelium. The underlying factor(s) that were responsible for graft failure in ~60% of optimized tubes remain unclear.

Optimally prepared tubes that were patent in the short term exhibited no thrombosis or infection and maintained patency for 7 days post-implantation. Explanted tubes displayed a similar level of granulation tissue inside and outside of the tube surface. Blood cells and fibroblasts in the inner surface of the tubes may have initiated the host response to the tube at the inner surface. The host response at the outer surface of the implanted tubes also resulted in generation of capillaries in the granulation tissue, which was continuous with a thin layer of new vascularized tissue that covered and integrated the tubes in the femoral cavity. Whether the original collagen tube was preserved in this implant reaction, or whether the surface of the tube was remodeled within the 1-week implant period, is unclear. The surface of the granulation layer inside the collagen tubes showed positive signal with CD31 immunohistochemistry. Our stain could not determine whether the CD31-expressing cells were the original RAOECs

lining the tubes, host rat-derived ECs that migrated from native femoral artery, and/or rat-derived platelets and monocytes. We speculate that the 5 mM genipin crosslinking condition provided the optimal environment of the tubes for the endothelium to withstand the surgical and in vivo fluid shear stresses and maintain endothelial function. It is also possible, however, that the host response from platelets and monocytes may be superior in the 5 mM genipin-treated tubes.

The results of the current study and our previous work show that preformed endothelium is important for patency in microsurgical vascular grafts (Li et al., 2017). In contrast, a preformed endothelium is not strictly required for long-term patency in vascular grafts of diameter ≥3 mm, where endothelium develops over time from a variety of sources (Dahl et al., 2011). This finding highlights one difference between design requirements for vascular grafts at the microsurgical versus larger size scales.

5 | CONCLUSIONS

The results described here are the first to demonstrate that, with optimized preparation conditions, it is possible to obtain long-term (7 days) patency with endothelialized dense collagen tubes as interpositional microsurgical grafts in the rat femoral circulation. The achievement of patent implants was only possible by tuning the scaffold crosslinking and culture conditions. Obviously, the patency of ~40% at one week post-implant needs to be improved. Moreover, the patency rate of implants beyond one week is unknown. Possible ways to improve endothelial stability include culturing seeded tubes for even longer times and/or under constant flow, coating the tube lumen with Type IV collagen or other matrix proteins for stronger endothelial cell adherence, and reducing the wall thickness of the collagen tube (while still maintaining sufficient strength) to further increase compliance. A similar dependence of graft patency on scaffold mechanics and culture condition may apply to other natural or synthetic tubular scaffolds under development, which may eventually lead to the engineering of robust grafts for vascular microsurgery.

ACKNOWLEDGMENTS

We thank Prof. Mark Grinstaff (Boston University) for interesting discussions and Dr. Yelena Akelina (Columbia University Medical Center) for microsurgical advice. This work was supported by award EB018851 and the Translational Research in Biomaterials training program (award EB006359) from the National Institute of Biomedical Imaging and Bioengineering.

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How to cite this article: Li X, Xu J, Bartolák-Suki E, Jiang J, Tien J. Evaluation of 1-mm-diameter endothelialized dense collagen tubes in vascular microsurgery. *J Biomed Mater Res*. 2020;108B:2441–2449. https://doi.org/10.1002/jbm.b.34576