

Introducing a 3-dimensionally Printed, Tissue-Engineered Graft for Airway Reconstruction: A Pilot Study

Todd A. Goldstein, MS^{1,2}, Benjamin D. Smith^{1,2},
 David Zeltsman, MD^{1,3}, Daniel Grande, PhD^{1,2}, and
 Lee P. Smith, MD^{1,4}

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Abstract

Objective: To use 3-dimensional (3D) printing and tissue engineering to create a graft for laryngotracheal reconstruction (LTR).

Study Design: In vitro and in vivo pilot animal study.

Setting: Large tertiary care academic medical center.

Subjects and Methods: A 3D computer model of an anterior LTR graft was designed. That design was printed with polylactic acid on a commercially available 3D printer. The scaffolds were seeded with mature chondrocytes and collagen gel and cultured in vitro for up to 3 weeks. Scaffolds were evaluated in vitro for cell viability and proliferation. Anterior graft LTR was performed on 9 New Zealand white rabbits with the newly created scaffolds. Three animals were sacrificed at each time point (4, 8, and 12 weeks). The in vivo graft sites were assessed via bronchoscopy and histology.

Results: The in vitro cell proliferation assay demonstrated initial viability of 87.5%. The cells proliferated during the study period, doubling over the first 7 days. Histology revealed that the cells retained their cartilaginous properties during the 21-day study period. In vivo testing showed that all animals survived for the duration of the study. Bronchoscopy revealed a well-mucosalized tracheal lumen with no evidence of scarring or granulation tissue. Histology indicated the presence of newly formed cartilage in the region where the graft was present.

Conclusions: Our results indicate that it is possible to produce a custom-designed, 3D-printed, tissue-engineered graft for airway reconstruction.

Keywords

3-dimensional, additive, manufacturing, printing, tissue, engineering, airway, reconstruction, laryngotracheal, graft

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Expansion laryngotracheal reconstruction (LTR) is typically performed with autologous cartilage. LTR may be performed with anterior and/or posterior cartilage grafting. For anterior graft LTR, cartilage may be harvested from thyroid ala, hyoid, auricle, or rib. For posterior graft LTR, costal cartilage is typically required because of its superior physical properties. Regardless of the donor site, autologous cartilage harvesting is associated with morbidity, including pain, cosmetic deformity, and additional surgical time. Furthermore, significant complications (eg, pneumothorax) are possible following costal cartilage harvest.

Several authors have attempted to address the issue of donor site morbidity by exploring alternative strategies for expansion LTR without the use of autologous cartilage. Sprecher reported good results performing anterior split laryngotracheoplasty on 10 patients with subglottic stenosis by use of a resorbable polylactic acid (PLA) miniplate to maintain the cricoid expansion without cartilage grafting.¹ Others have explored the use of titanium mesh, plastipore, B-tricalcium phosphate, and hyaluronic acid in animal models.^{2–4} None of these techniques have gained widespread acceptance. Several authors have demonstrated success with tissue-engineered cartilage for use in various animal models of LTR.^{5–7} While not directly related to LTR surgery, there is a body of work exploring tissue engineering strategies for tracheal replacement surgery.^{8–10} Furthermore, this concept of a completely synthetic tissue engineered tracheal replacement has been explored in limited human clinical testing.¹¹

¹Hofstra North Shore-LIJ School of Medicine, Hempstead, New York, USA

²Orthopaedic Research Laboratory, Feinstein Institute of Medical Research, Manhasset, New York, USA

³Department of Cardiovascular and Thoracic Surgery, Hofstra North Shore-LIJ School of Medicine, Hempstead, New York, USA

⁴Division of Pediatric Otolaryngology, Steven and Alexandra Cohen Children's Medical Center, New Hyde Park, New York, USA

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Corresponding Author:

Lee P. Smith, MD, Steven and Alexandra Cohen Children's Medical Center, 430 Lakeville Road, New Hyde Park, NY 11042, USA.

Email: LSmith8@nshs.edu

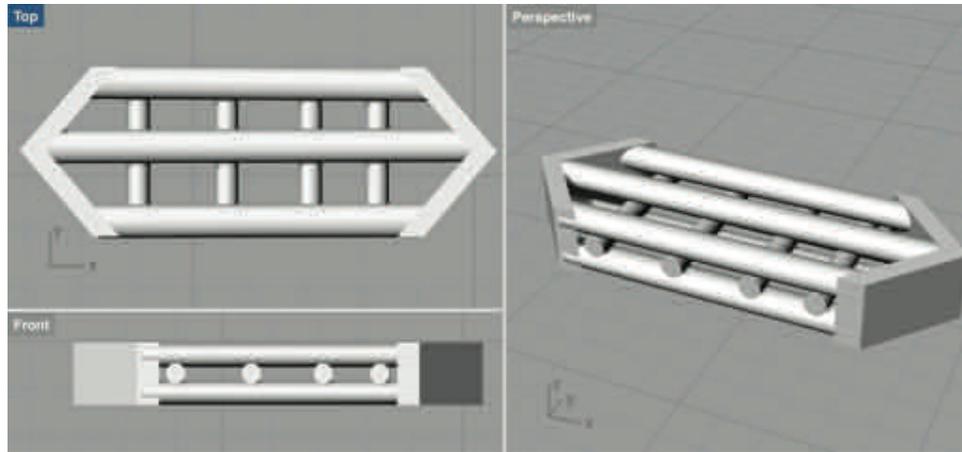


Figure 1. Laryngotracheal reconstruction scaffold design. Front, top, and perspective views of the laryngotracheal reconstruction computer-aided design with multiple channels shown to allow for cellular incubation and growth.

Recently, 3-dimensional (3D) printing or additive manufacturing techniques have been employed to fabricate a custom-designed external splint for tracheomalacia.¹² This technique was used successfully in at least 1 pediatric patient with severe airway malacia.¹³ Three-dimensional printing and tissue engineering have the potential to produce tissue and organ replacement. Previous work from our laboratory has demonstrated the potential to fabricate a custom tracheal ring composed of living functional chondrocytes. In this experiment, we combined 3D printing and tissue engineering to produce a living biological graft for LTR and tested that graft in an animal model.

Material and Methods

Design and Printing of Scaffold

A 3D computer-aided design (CAD) model of an anterior LTR graft was created with the Rhino3d Wenatchee-OsX CAD designer. The design was modeled to produce a graft that was 8 mm in length, 3 mm in width and 1.2 mm in depth. The graft was designed with multiple spaces to allow for cellular incubation, and it included spaces on each side for sutures (**Figure 1**). The design template was optimized for 3D printing in MakerWare software (MakerBot, Brooklyn, New York). Scaffolds were 3D printed with PLA on a MakerBot Replicator Desktop Printer.

Isolation and Culture of Cells

Chondrocytes were harvested from the trachea of 1 New Zealand white rabbit. The rabbit was placed in dorsal supine position, and a vertical midline incision was made through skin. The anterior cervical strap muscles were divided along the midline raphe. The anterior tracheal wall was identified, and 4 tracheal rings were removed. The cartilaginous portions of the rings were isolated and separated from the surrounding tissues. The cartilage samples were washed several times with an antibiotic-antimycotic solution (Cellgro, Manassas, Virginia) and minced. The minced cartilage was added to the collagenase solution (2.4 mg of NB4 collagenase per milliliter of DMEM; Serva Electrophoresis,

Heidelberg, Germany) and digested at 37°C for 4 hours in a spinner flask. After digestion, the cell suspension was sieved twice through a 100- μ m cell strainer. The cell suspension was centrifuged at 1500 rpm for 10 minutes. The cells were counted and plated in culture media at a density of 10,000 cells/cm². Cells were expanded out 3 passages in culture media (DMEM/F-12 50/50 1 \times media; Cellgro, Manassas, Virginia) with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution (Cellgro) and added into the gel. Samples were incubated at 37°C, 5% CO₂, and 95% humidity for the duration of their time points, and the media was changed biweekly.

Gel Preparation

Collagen type 1 gel was produced by combining 8 mL of PureCol Purified Bovine Collagen Solution (3.1 mg/mL; Advanced BioMatrix, San Diego, California) with 1 mL of 10X RPMI (Sigma-Aldrich, St Louis, Missouri). The pH was neutralized by adding 1M NaOH added dropwise. The gel was used to resuspend cell pellets when needed.

Cell Culturing

Cells were suspended in 4 mL of type 1 collagen gel, as previously described. Approximately 300,000 cells (50 μ L of the suspension) were injected into each scaffold and placed in culture media (DMEM/F-12 50/50 1 \times media; Cellgro) with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution (Cellgro). Samples were incubated at 37°C, 5% CO₂, and 95% humidity for the duration of their time points, and the media was changed biweekly.

Cell Proliferation Assay

Three-dimensionally printed cell-seeded scaffolds were analyzed for cell proliferation with the MTS assay (Promega, Madison, Wisconsin). At each time point, scaffolds were removed from their cell culture media, and three 100- μ L aliquots of each sample's incubation medium were transferred to a 96-well plate. Cells were then counted by measuring their absorbance against a standard curve measured

at 492 nm via an ELX800 plate reader (Biotek, Winooski, Vermont).

Cell Labeling

Chondrocytes were labeled (Calcein AM; Life Technologies, Waltham, Massachusetts) prior to printing to visualize distribution throughout the scaffold. Cells were resuspended in type 1 collagen gel and injected into the scaffold with a 22-gauge flat needle. The sample was photographed fluorescently with a 490-nm excitation filter and a 520-nm emission filter.

Histology

At 1, 7, 14, and 21 days, samples were fixed in 10% Millonig's buffered formalin. The specimens were embedded in paraffin, and serial 5- μ m sections were cut in a coronal plane, mounted on glass slides, and stained with hematoxylin and eosin and safranin O/fast green. The samples were examined with a bright-field microscope, and digital images were captured.

In Vivo Studies

Anterior graft LTR surgery was performed on 9 New Zealand white rabbits with the same chondrocytes harvested for the in vitro studies. Ketamine (20-60 mg/kg, intramuscular or subcutaneous) was given in the home cage for sedation. Preemptive analgesia was given prior to surgery by injecting buprenorphine (0.05-0.1 mg/kg) and carprofen (4-5 mg/kg) subcutaneously 15 to 20 minutes before anesthesia induction. Rabbits were then induced and maintained with isoflurane (1%-5%) plus oxygen (95%), administered by mask. The rabbits were placed in dorsal supine position after induction of anesthesia. The rabbit was prepared and draped in the usual manner with 3 alternating applications of povidone iodine solution and alcohol. One milliliter of 1% lidocaine was injected into the subcutaneous tissue in a vertical midline fashion. A 3-cm vertical midline incision was made through skin. The anterior cervical strap muscles were divided along the midline raphe. The anterior tracheal wall was identified. One 4-0 Vicryl (Ethicon, Somerville, New Jersey) stay suture was placed on either side of midline through the third and fourth tracheal rings. A vertical midline tracheotomy was made involving the third through fifth tracheal rings (8 mm in length). Two 4-0 PDS sutures (Ethicon) were placed on either side of the graft in a simple interrupted fashion to secure the graft into the tracheal defect. A small amount of Tisseel fibrin glue (0.3 mL; Baxter International, Deerfield, Illinois) was utilized to secure the graft in place. The strap muscles and skin were reapproximated with 4-0 Vicryl sutures in an interrupted fashion. Postoperatively, carprofen (4-5 mg/kg) was given once daily for 3 days. Three rabbits each were sacrificed at 4, 8, or 12 weeks following surgery. This study was approved by the institutional animal care and use committee at the Feinstein Institute for Medical Research.

Bronchoscopy

At necropsy, bronchoscopy was performed with a 4-mm Hopkins rod telescope with video recording and photodocumentation (Karl Storz, El Segundo, California). Images

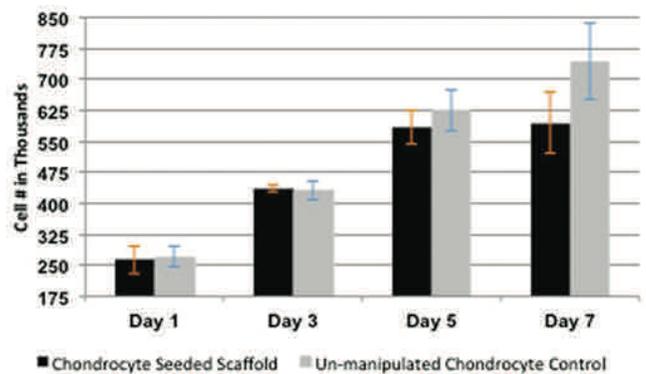


Figure 2. MTS cell proliferation assay over a 7-day period. Similar proliferation rates for cells seeded in the scaffold as compared with unmanipulated controls.

were qualitatively evaluated for the presence of granulation tissue or scarring.

Histology

Following sacrifice, the tracheal specimens were fixed in 10% Millonig's buffered formalin. The specimens were embedded in paraffin, and serial 5- μ m sections were cut in a coronal plane, mounted on glass slides, and stained with hematoxylin and eosin and safranin O/fast green. The samples were examined with a bright field microscope, and digital images were captured.

Statistical Analysis

Proliferation data were examined by a 2-tailed *t* test comparing the growth rates of unmanipulated control chondrocytes versus chondrocytes in the 3D-printed scaffold; the *t* test was performed with StatPlus software (AnalystSoft, Walnut, California; $\alpha = 0.05$).

Results

Our results indicate that it is possible to produce a custom-designed, 3D-printed, tissue-engineered graft for airway reconstruction. Cell proliferation assay of 32 samples yielded an average initial viability of 87.5% at 24 hours. Cell number increased over 7 days to approximately twice the initial concentration (**Figure 2**). Cells grown in the 3D-printed scaffolds were compared with 32 control sample cells grown in identical culture conditions; *t* test showed no significant difference in proliferation rate ($P = .42$).

At 24 hours, postincubation histology and cell labeling displayed equal distribution of cells throughout the scaffold (**Figure 3**). The cells retained their characteristic round shape and displayed an equal distribution throughout the 21-day study period. Numerous islands of safranin O-positive staining were displayed within the scaffold, and the quantity of staining increased throughout the 21-day period. Islands of safranin O-positive staining increasing over time are indicative of chondrocytes maintaining their biological properties throughout the experiment.

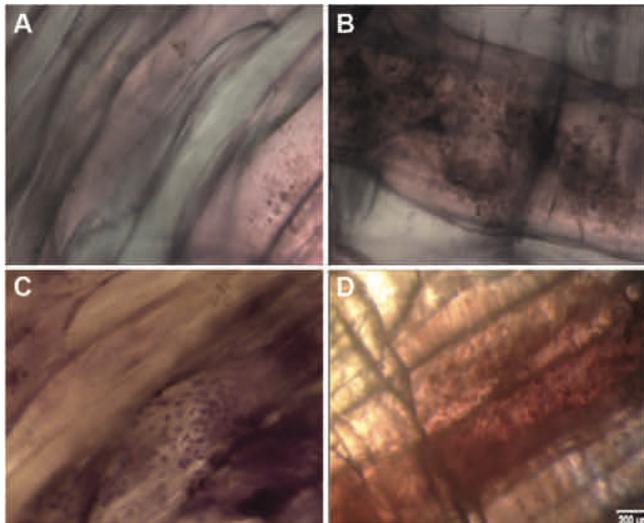


Figure 3. Phase-contrast microscopy (10 \times) of construct. Hematoxylin and eosin staining at (A) day 1 and (B) day 21. Safranin O/fast green staining at (C) day 7 and (D) day 21.

Our results also demonstrate the feasibility of implanting these grafts in vivo in an animal model of airway reconstruction. All 9 animals (100%) survived for the duration of the study. Bronchoscopy revealed that the endolumen of the trachea was well mucosalized, with no evidence of scarring or granulation tissue in any rabbit at any of the time points tested (**Figure 4**).

Histology was performed in the region of the grafts for all animals. Our results indicate that newly formed cartilage was present in the location where the grafts were implanted. At 8 weeks (**Figure 5A**), early deposition of an aggrecan-rich cartilage matrix can be seen, although it is still not as mature as the host tracheal cartilage. At 12 weeks, note the increased content of aggrecan present in the cartilage extracellular matrix (**Figure 5B**). There was no evidence of significant inflammation, foreign body reaction, or granulation tissue in any of the animals at any of the time points.

Discussion

Three-dimensional printing (or additive manufacturing) involves the custom fabrication of a 3D solid object from a digital design. In most cases, CAD software is used to create a virtual model, and the printer is used to bring that model into the physical world. Three-dimensional printing has already been used as a training tool in various aspects of medicine and surgery and for surgical planning for cardiac surgery and plastic surgery.^{14,15} If this technology can be adapted into the realm of airway surgery, 3D printing could allow for individualized constructs with ideal biomechanical properties and physiologic function. Indeed, surgeons have already attempted to address airway malacia by means of external airway splinting with biodegradable 3D-printed scaffolds without chondrocytes. This technique has been applied to at least 1 pediatric patient in severe respiratory distress for whom no other options were available.¹³

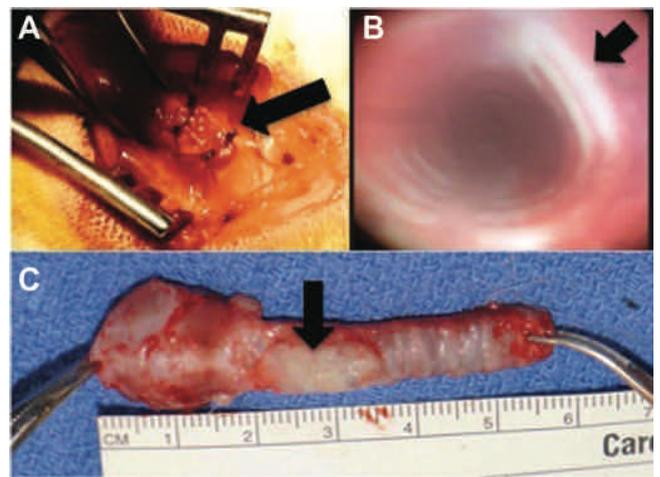


Figure 4. Photographs of typical graft during and after in vivo placement in rabbit: (A) intraoperative view, (B) bronchoscopic view at 4 weeks, (C) ex vivo rabbit trachea with graft at 4 weeks. Arrows indicate the location of the graft (A and C) and the lumen of the trachea without granulation tissue or scarring (B).

Expansion LTR typically requires autologous cartilage harvest, which may be associated with donor site morbidity. Several authors have addressed this challenge testing a variety of nonresorbable biomaterials in various experimental models.^{4,16-19} One major concern with nonresorbable biomaterials is their potential for inducing a chronic inflammatory response with granulation tissue and extrusion. With this in mind, several groups have explored the use of resorbable biomaterials for airway surgery. Javia and Zur detailed their technique using a commercially available resorbable miniplate as an external lateral buttress in patients undergoing LTR surgery who had unexpected airway malacia in addition to stenosis.²⁰ Another group used similar resorbable miniplates to secure autologous costal cartilage in place in a canine model of anterior graft LTR.²¹ Klein et al tested a commercially available combination PLA and polyglycolic acid scaffold in a rabbit model of LTR.²² Their study did not involve cell seeding of the scaffold or autologous cartilage. Their results showed that rabbits were able to survive the duration of the study and that the reconstructed airways were airtight with adequate mechanical properties. Following resorption of the scaffold, however, most animals were left with a fibrous bridge in the airway without evidence of cartilage formation.²² The presence of a fibrous bridge alone in the region of the reconstruction suggests that this approach may not produce adequate long-term results and may not translate well into larger animals.

Hubbell et al tried using irradiated homologous cartilage in a rabbit model of LTR; their results demonstrated high rates of resorption, and they advocate using autologous cartilage in spite of its donor site morbidity.²³ More recently, tissue engineering solutions for airway reconstruction have been examined. Kamil et al demonstrated success creating a tissue-engineered graft for LTR by mixing chondrocytes from harvested auricular cartilage with a synthetic polymer.⁷ The mixture was then implanted subdermally and allowed

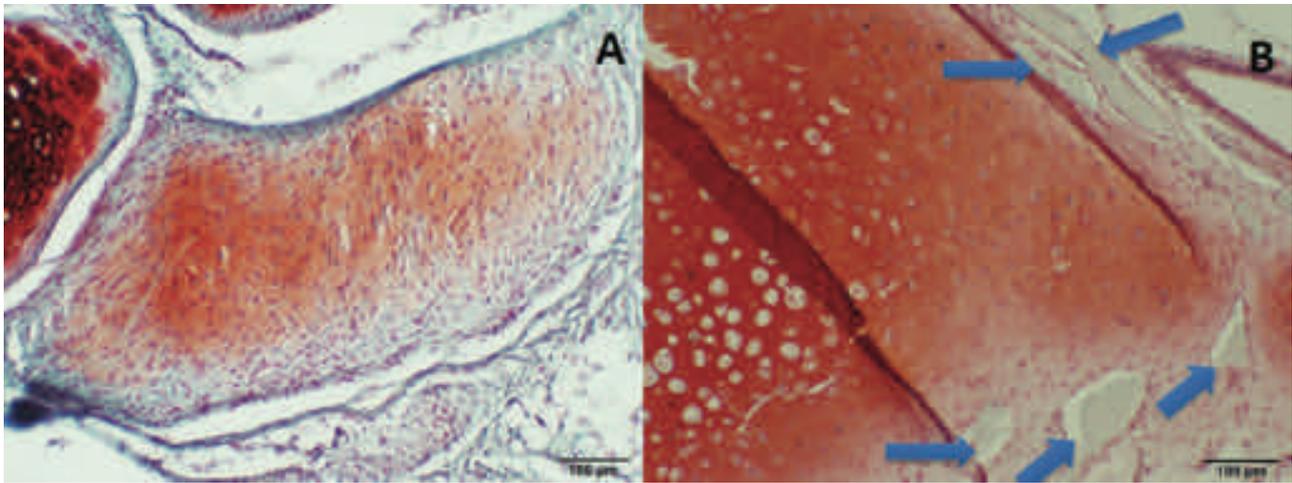


Figure 5. In vivo histology: (A) de novo cartilage formation at 8 weeks and (B) neocartilage formed at 12 weeks. The original polylactic acid construct is still present (arrows). 100 \times original magnification. Safranin O/fast green.

to mature in vivo for 8 to 12 weeks. The newly created grafts were then harvested and used to perform anterior graft LTR on 3 pigs.⁷ Weidenbecher et al tested a hyaluronic acid scaffold seeded with autologous auricular cartilage cells in a rabbit model of LTR.³ Their results showed a significant inflammatory response to the scaffold. Three years later, the same group produced a tissue-engineered cartilage graft for LTR without the use of a scaffold and demonstrated some success using a rabbit model of LTR.²⁴

The combination of living tissue, whose proliferation rates are comparable to native cells, and a 3D-modeled patient-specific graft may provide a tissue-engineered graft for airway surgery that can be customized for each patient's needs. In this study, we demonstrate the ability to fabricate a 3D-printed biodegradable PLA scaffold containing proliferating chondrocytes. Our data indicate that the cells within the scaffold may remain viable, proliferate, and appear to retain their biological function by synthesizing extracellular matrix. When tested in an in vivo model of LTR, our animals survived the surgical procedure. The airways showed a minimal inflammatory response to the scaffold with cartilage formation and mucosalization of the lumen. These results indicate that new tracheal cartilage tissue might be able to be achieved by transplantation of 3D-bioprinted chondrocytes within a LTR construct. Chondrocytes survived the rigors of 3D printing within a bioink and were metabolically capable of reconstituting functional extracellular matrix with a high aggrecan content. The long-term in vivo results of our strategy are unclear, and further study would be necessary to evaluate its potential. Furthermore, we used tracheal cartilage as a donor site. In hypothetical clinical practice, auricular cartilage would most likely be used as a donor site. Further study would be required to show that auricular cartilage would be adequate.

While not specifically researching a LTR model, several groups have explored tissue engineering solutions for tracheal replacement and reconstruction.^{8-10,25} Using similar biomaterials, 2 groups were able to produce a biodegradable

tracheal replacement scaffold that was seeded with mature chondrocytes, exposed to exogenous growth factors, and tested successfully as a partial tracheal replacement in a rabbit model.^{9,10} Recently, several groups have actually begun to combine tissue engineering and 3D printing to produce tracheal replacement grafts.^{8,25} All of these strategies have potential drawbacks, including complex scaffold fabrication processes and the use of stem cells and/or growth factors, which may have an unknown long-term phenotype and neoplastic potential.²⁵

Our approach involves the creation of a CAD design that is optimized through commercially available software and printed on an inexpensive and readily available device. PLA was chosen to fabricate the scaffold, as it is already approved by the Food and Drug Administration for various uses. Furthermore, the scaffolds are seeded with mature chondrocytes, which appear to survive and retain their biological function. We believe that the use of mature chondrocytes as opposed to stem cells would serve to minimize the long-term malignant potential.

One drawback of this study is the lack of a control group in the in vivo segment. The in vivo portion of the study was intended as a proof of concept, and we wanted to minimize the number of animals sacrificed. Klein et al previously evaluated the in vivo response of a similar biodegradable scaffold that was not seeded with chondrocytes.²² Their results indicated that a fibrous bridge was present in the airway after scaffold degradation, while our results demonstrate the presence of cartilage tissue.

Conclusions

This study demonstrates the potential to fabricate a 3D-printed, tissue-engineered graft for LTR. That graft retains its cartilaginous properties in vitro and in vivo and may have applications in airway reconstruction. Our technique employs commercial products that are inexpensive and readily available. The combination of 3D printing and tissue

engineering holds promise for solving many clinical problems in airway surgery.

Author Contributions

Todd A. Goldstein, data analysis, drafting, final approval, accountability for all aspects of the work; **Benjamin D. Smith**, data analysis, drafting, final approval, accountability for all aspects of the work; **David Zeltsman**, data analysis, drafting, final approval, accountability for all aspects of the work; **Daniel Grande**, data analysis, drafting, final approval, accountability for all aspects of the work; **Lee P. Smith**, data analysis, drafting, final approval, accountability for all aspects of the work.

Disclosures

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