Novel Therapy for Acquired Tracheomalacia with a Tissue-Engineered Extraluminal Tracheal Splint and Autologous Mesenchymal-Derived Chondrocytes

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Abstract

Introduction
Acquired tracheomalacia (ATM) is characterized by a loss of structural strength of the tracheal framework, resulting in airway collapse during breathing. Near half of the patients undergoing prolonged invasive mechanical ventilation will suffer tracheal lesions. Treatment for ATM includes external splinting with rib grafts, prosthetic materials, and tracheal resection. Failure in the use of prosthetic materials has made reconsidering natural origin scaffolds and tissue engineering as a suitable alternative.

Objective
To restore adequate airway patency in an ovine model with surgically-induced ATM employing a tissue-engineered extraluminal tracheal splint (TE-ETS).

Methods
In the present prospective pilot study, tracheal rings were partially resected to induce airway collapse in 16 Suffolk sheep (Ovis aries). The TE-ETS was developed with autologous mesenchymal-derived chondrocytes and allogenic decellularized tracheal segments and was implanted above debilitated tracheal rings. The animals were followed-up at 8, 12, and 16 weeks and at 1-year postinsertion. Flexible tracheoscopies were performed at each stage. After sacrifice, a histopathological study of the trachea and the splint were performed.

Results
The TE-ETS prevented airway collapse for 16 weeks and up to 1-year postinsertion. Tracheoscopies revealed a noncollapsing airway during inspiration. Histopathological analyses showed the organization of mesenchymal-derived chondrocytes in lacunae, the proliferation of blood vessels, and recovery of epithelial tissue subjacent to the splint. Splints without autologous cells did not prevent airway collapse.

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Introduction

Acquired tracheomalacia (ATM) is characterized by a loss of structural strength of the anterolateral tracheal framework. It produces a dynamic obstruction due to airway collapse during breathing, causing inefficient secretion clearance, stridor, respiratory distress, and even death. Intubation, trauma, neoplasms, and autoimmune diseases are frequent causes. The true prevalence of the disease is unknown, but in patients undergoing tracheoscopy, the incidence ranges from 1 to 23%. In cases of severe tracheomalacia, mortality is as high as 80%. Current treatment options include management with continuous positive airway pressure (CPAP), extraluminal splinting with rib grafts or prosthetic materials, intraluminal silicone, metal stents, and tracheal resection with end-to-end anastomosis. These are either partial solutions or are highly morbid and are not always suitable for critically ill patients. In the field of tissue engineering, scaffolds should be biocompatible, biodegradable, nonimmunogenic, and possess biomechanical strength. So, current trends focus on tracheal substitution, which might be accomplished through allotransplantation, autologous tissue reconstruction, aortic homograft reconstruction, and tissue engineering. High hopes were placed on the latter, but premature clinical translation and failure were a setback. Further preclinical studies are needed to ensure safety and efficacy before clinical translation. Natural-origin scaffolds have been reconsidered as an alternative to ensure safety and efficacy before clinical translation. Natu-

Methods

Ethics

The Institutional Animal Care and Use Committee (RLFSA: AUT-B-A-0315–20) approved all procedures under protocol number INRLGI-50/14. Institutional collaboration included the Otolaryngology Department, the Biotechnology Laboratory, and the animal surgery unit for animal care.

Chondrogenic Differentiation and Generation of a Novel TE-ETS

Ovine mesenchymal cells (oMSCs) were obtained from epiploic adipose tissue. The cells were isolated by digestion with modified Eagle Medium/Nutrient Mixture F-12 [DMEM-F12] 10 ng/mL transforming growth factor β1 [TGF-β1], 1.0 mg/mL insulin, 10 mM dexamethasone, 20 mg/mL ascorbic acid, 1% FBS and 1% penicillin-streptomycin) for 21 days. Differentiation was evaluated in vitro and performed at large-scale culture for splint implantation following the same protocol. Cadaveric heterologous tracheal segments (5–8 cm) were treated with 2 to 6 decellularization cycles (1% trypsin-EDTA for 5 hours at 37°C, 10% guanidine-Tris overnight, sonication in 4% sodium deoxycholate for 3 hours, 1% sodium dodecyl sulfate overnight, and 10 mg/mL DNAse type 1 for 2 hours at 37°C). The DNA content was evaluated in histological sections by nucleus staining with DAPI. The mechanical properties of heterologous tracheal segments were continuously evaluated by participant surgeons. For novel TE-ETS generation, decellularized segments were ensheathed with oMS-DC embedded at 4 × 10⁷ cells/mL in medical-grade fibrin hydrogels as the cell carrier biomaterial. Differentiated cells were applied onto the surface of the splint and allowed to polymerize during implantation surgery.

Animal Care and Perioperative Technique

Sixteen 12-month-old female Suffolk sheep (Ovis aries) with an average weight between 25 and 60 kg were used. In all procedures, acepromazine maleate (0.05 mg/kg IV) and propofol (5 mg/kg, IV, PLOFED-20) were administered for sedation before endotracheal intubation. General anesthesia was maintained with isoflurane 3.0% and 3 l/min oxygen. Perioperative care consisted of an intramuscular (IM) antibiotic, either enrofloxacin (5.0 mg/kg for 5 days) or clindamycin–gentamycin (2.5 mg/kg for 5 days), the IM analgesic dipyrone (50 mg/kg for 5 days), and the IM antihistamine diphenhydramine (1.0 mg/kg single dose). The animals were extubated and moved to individual pens for close surveillance and antibiotic therapy until the 4th day, when they rejoined the herd.

Conclusion

It is possible to treat acquired tracheomalacia with TE-ETS without further surgical removal since it undergoes physiological degradation. The present study supports the development of tissue-engineered tracheal substitutes for airway disease.
rings was resected until airway collapse was visible. The mucosa was not injured. To avoid fibrosis, the resected area was covered with a 2.0 mm-thick medical-grade silicone sheet. Tracheoscopies were performed to confirm airway collapse.\(^{15}\)

**Surgical Insertion of TE-ETS, Postoperative Evaluation, and Sacrifice**

Four weeks after ATM induction, the TE-ETS was surgically implanted into each subject through a standardized procedure. The trachea was exposed via a midline cervical incision, the silicone sheet was removed, and the area was debrided and covered with a layer of fibrin hydrogel-embedded differentiated cells. The splint was fitted and sutured to the ATM area and covered with the second layer of cells. The experimental models (\(n = 16\)) were divided into 3 groups according to the follow-up time: Group I (8 weeks postinsertion [WPIs]), Group II (12 WPIs), and Group III (16 WPIs). One specimen was kept in observation for 1 year after insertion. To analyze the role differentiated chondrogenic cells play in tissue regeneration, scaffolds without cells were implanted in three subjects, which were distributed evenly in Groups I, II, and III. During follow-up, the animals were monitored for clinical signs of inflammation or rejection and serial tracheoscopies were performed. After completing the pre-established follow-up period, the models were sacrificed by IV pentobarbital overdose (70 mg/kg), and the TE-ETS were removed. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and histological slices were stained with safranin O for analysis of the cartilage and proteoglycans.

**Statistical and Cellular Analysis**

Group comparisons were made by t-tests (2 groups) or by one-way analysis of variance (ANOVA) with posthoc test (> 2 groups). P-values < 0.05 were considered significant. All quantitative data are expressed as means and standard deviations (SDs). Cell percentages were calculated by counting individual cells in at least five fields per experiment. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

**Results**

**Extraluminal Tracheal Splint in Biocompatible with Autologous Mesenchymal Cell-Derived Chondrocytes**

Ovine adipose tissue allowed isolation of adherent cells from stromal-vascular fraction (SVF) 24 hours after cell seeding. Primary adipose-derived oMSCs exhibited fibroblast morphology (\(\sim\) Fig. 1a) and colony-forming capacity after 2 days in monolayer culture. By day 5, the colonies had between 50 and 250 cells/colony (\(\sim\) Fig. 1b). Cell viability was 95.7 ± 1.1% after 7 days in culture (\(\sim\) Figs. 1c and g). Cell proliferation, measured by the expression of Ki67, was detected in 7.5% of the cells in the early phase of primary culture (\(\sim\) Figs. 1d and h). Ovine mesenchymal cells-derived chondrocytes changed from spindle to polygonal shape and displayed aggregate formation nodules that increased in size over the differentiation period. Glycosaminoglycans (GAGs) in the cell cytoplasm and matrix stained positively by Alcian blue (\(\sim\) Figs. 1e). Nonstimulated cells did not display significant changes, aggregation, or positive staining, but cell growth was similar to that of oMSCs (\(\sim\) Fig. 1f). DAPI staining was performed to verify the absence of DNA content in cadaveric heterologous tracheal segments. Staining the nontreated tracheal cartilage detected DNA in the chondrocyte nuclei and in the connective tissue that ensheaths the cartilage (\(\sim\) Fig. 2a). After two cycles of decellularization, the DNA content gradually reduced (\(\sim\) Figs. 2b and c). After three cycles, the DNA content was degraded, leaving residue on the inner parts of the tissue that was positive to DAPI staining (\(\sim\) Fig. 2d). Chondral lacunae were visibly absent of chondrocytes or nuclei compared with the untreated trachea (\(\sim\) Fig. 2e). The tracheal segments underwent a considerable reduction of proteoglycans, but the extracellular matrix (ECM) structure of cartilage was maintained except for the connective tissue and soft muscle that joins the cartilaginous rings (\(\sim\) Fig. 2f). In general, three decellularization cycles resulted in the elimination of...
undesired connective tissue layers while maintaining the rigidity and flexibility of the tracheal segment, allowing manipulation and size adjustment. Further cycles led to a loss of ECM integrity, and even separation of cartilaginous rings, compromising the functionality of the extraluminal splint (►Fig. 2i-k). Ovine mesenchymal cells-derived chondrocytes were embedded in fibrin hydrogels to allow cell growth and cellular adhesion to the splint scaffold. It was possible to observe viable chondrocytes developing a polygonal shape similar to that displayed in the high-density cultures. The viability of the cells in the hydrogel-embedded chondrocytes was 94.7% after 7 days of culture (►Fig. 2h and g).

**Partial Cartilage Resection Causes Airway Collapse in an Ovine Model**

A standardized animal model of induced tracheomalacia was obtained via full-thickness removal of 70% of the circumference of 5 tracheal rings, avoiding mucosal injury (►Fig. 3a, 3b). Removal of five rings caused dyspnea at rest and audible stridor, hence the number of tracheal rings resected was reduced to four, which was still sufficient to produce airway collapse (►Fig. 3c, 3d). Dyspnea with exertion was evident in all subjects (►Fig. 3d and e), so it was considered an appropriate tracheomalacia model. Before the TE-ETS was surgically implanted, the persistence of airway collapse in each tracheomalacia model was verified with a tracheoscopy.

**Engineered Extraluminal Splint Restores Airway Patency and Promotes Tissue Regeneration**

When the TE-ETS was implanted above the malacic area, it adopted the tubular shape of the trachea and was easy to handle (►Fig. 4a). Fibrin-embedded oMS-DC favored fixation of the TE-ETS to the malacic site (►Fig. 4b). None of the animals had clinical signs of inflammation or rejection, including fever, anorexia, or abnormal behavior after surgery. All treated animals (n = 16) survived after insertion of the TE-ETS. A final tracheoscopy performed before sacrifice showed no airway collapse during inspiration, and lumen constriction due to fibrosis was < 10%. In-vivo airway collapse was not evident even after applying mild manual pressure over the treated area. In some animals, fibrotic rings formed around the splint, but the airway was not compromised (►Fig. 4c). The TE-ETS showed vascular organization, allowing the airway to recover function. All splints were found in place, surrounded by fibrous tissue; this made the splint harder than when implanted, but it was still elastic (►Fig. 4d and e). No major macroscopic differences were seen between the splints amongst the different groups (►Fig. 4f).

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**Fig. 2** Decellularization of extraluminal tracheal splints and hydrogel culture of oMSC-derived chondrocytes. DNA content in histological slices of untreated heterologous tracheal segments (a). After decellularization, the DNA content gradually reduced (b-c-d). Safranin O staining revealed proteoglycans found in ECM in native tracheal cartilage (e) and their reduction after three decellularization cycles (f). Ovine MSC-derived chondrocytes embedded in fibrin hydrogels showed 94.7% cell viability after 7 days culture (h). Decellularization resulted in the elimination of undesired connective tissue layers while maintaining rigidity and flexibility of the tracheal segment allowing manipulation and size adjustment (i-k). Scale bars 50 µm (a-f) and 100 µm (g).
Animals implanted with a decellularized scaffold without oMS-DC showed persistence of tracheal collapse during inspiration. After sacrifice, the splints induced a low inflammatory infiltrate, displayed a breach from the contiguous connective tissue without edema, and had unstable tissue constitution adjacent to the lesion (►Fig. 5 panel a). After 8 WPIs, TE-ETSs were properly positioned above the ATM area and subjacent tissue showed areas of mineralization accompanied by an inflammatory infiltrate with predominance of lymphocytes and histiocytes. Fibrosis zones contained abundant deposits of collagen mainly constituted by fibroblasts with barely perceptible nuclei. Safranin O staining revealed adequate decellularization of the external splint compared with tracheal cartilage (►Fig. 5 panel b). After 12 WPIs, airway collapse decreased but was still detectable. Staining revealed that the inflammatory infiltrate resolved and there were no areas of osseous metaplasia or granulomas. Areas with steatonecrosis and ossification were not considerable, and tissue subjacent to the extraluminal tracheal splints showed a dense constitution. The TE-ETS had no proteoglycans, following previous decellularization findings. It is also noteworthy that we detected organization of oMS-DC in lacunae and proliferation of blood vessels in tissue subjacent to the TE-ETS (►Fig. 5 panel c). For models sacrificed at 16 WPI, tracheal collapse significantly decreased. Granulomas and considerable inflammatory infiltrate were absent, while mineralization, cytonecrosis, osteonecrosis, ossification, or metaplastic changes also resolved. Tissue underlying implanted areas presented neoinformation of ECM, containing oMS-chondrocyte aggregates (►Fig. 5 panel d). Remarkably, in the animal that was kept under observation for 1 year after splint insertion, tracheal collapse was not detected, and the tracheal diameter resembled that of uninjured animals. Slow degradation of the splint scaffold was noticeable; nevertheless, a remarkable regeneration of ECM in ATM areas was observed, which restored the mechanical strength of the trachea. The respiratory epithelium suffered changes after ATM induction, but it recovered in both models implanted with the acellularized splints and those that received the TE-ETSs. The vascularization, reconstitution, and inner layer of the tracheal mucosa were most evident 1 year postinsertion (►Fig. 5 panel e). In all cases, autologous cells promoted adherence and tissue regeneration between the TE-ETS and ATM zones.

Discussion

More traditional treatments for ATM, such as intraluminal splints, have had discouraging results. Complex surgical procedures are associated with a high risk of complications, such as anastomosis, dehiscence, and wound infection. Tracheal replacement methods that do not involve tissue engineering include allogeneic transplantation, autologous tissue reconstruction, and aortic homograft reconstruction; however, currently, there is still no ideal tracheal substitute. Unresolved problems include mucociliary clearance, poor vascular nutrition to the neotrachea, and the need for immunosuppression. In the present study, we were able to overcome these issues by developing a TE-ETS that has the ideal splint properties of being malleable, nonimmunogenic, and absorbable, as well as promoting neotissue formation.
The two main components are the splint and the oMS-derived chondrocytes seeded to the splint. The former provides support, avoids mechanical stress, and reduces airway collapse, while the latter promotes the formation of connective tissue and cartilage islets that improve mechanical support in the ATM area. Once the splint is absorbed, the newly developed cartilage remains and prevents airway collapse. Ovine mesenchymal cells that met the criteria described for mesenchymal stromal cells (MSC) by the International Society for Cellular Therapy\textsuperscript{17} were obtained. These cells achieved the multipotency potential reported for oMSC from peripheral blood,\textsuperscript{18} human adipose-derived MSCs, and endometrium.\textsuperscript{19} Chondrogenic differentiation potential based on GAGs production and cell proliferation rate were also comparable to human MSC.\textsuperscript{20} Hence, ovine adipose-derived MSCs retain the ability to differentiate and fulfill requirements for tissue engineering models. The mechanical properties of the decellularized trachea have been demonstrated to have similar biomechanical profiles to the native trachea.\textsuperscript{21} Another advantage of decellularized trachea is that their microstructure plays an important role in neotissue formation through chemoattraction, cell support, and signaling.\textsuperscript{22} Most studies have their endpoints established in < 90 days, including poly-$\varepsilon$-caprolactone (PCL) 3D-printed materials, whereas our sheep were kept alive up to 1 year after implantation. This longer period of observation allowed us to explore how the TE-ETS integrated into the tissue and the risk of producing stenosis on a long-term basis. Additionally, most of the trials using PCL 3D-printed materials were completed before absorption, a process that takes between 2 and 3 years. Therefore, it is not possible to analyze if airway collapse could be prevented solely by neotissue formation after the splint was absorbed.\textsuperscript{23} It will be interesting to compare the outcomes of PCL versus decellularized splints, with and without cellular components, especially on a long-term basis. An alternative to produce the splint was 3D printing; however, despite progress made with 3D printing technology, it is recognized that the development of improved biomaterials and imaging techniques is still required to increase the applicability of 3D printed devices.\textsuperscript{24}

The combination of biological, chemical, and physical agents for the disruption of cell membranes improved the dynamic decellularization of allogenic tracheal segments after three cycles while preserving the main constituents of ECM. This process removes adventitia, blood vessels, and internal mucosal folds. This is relevant because the MHC molecules that are responsible for immunogenicity reside in noncartilaginous tissue.\textsuperscript{25} Based on the fact that elimination of heterologous DNA reduces the immunological response of the host,\textsuperscript{26} our strategy included the addition of autologous oMS-chondrocytes to minimize immune response. As proof, the animals showed no signs of rejection after implanting the splints, despite receiving no immunosuppression. The scaffold can be adapted to lesion requirements at the moment of surgical implantation. In our model, hydrogel-embedded oMS-chondrocytes formed a cellular layer onto the malacic site, facilitating fixation and immobilization of the splint to the debilitated area. A logistical advantage of this model is that cells can be taken directly from the cell culture facility to the operating room, where they are seeded. Another advantage is that production times are optimized; for example, a TE-ETS is fully developed for implantation in < 8 weeks, but when previously differentiated autologous cells are used, the estimated development time is 2 weeks.

Traditionally, rabbits have been used as models of tracheal airway collapse because the diameter and elasticity\textsuperscript{27} of their trachea are similar to that of the human pediatric trachea.
However, in adulthood, rabbits have almost complete tracheal rings, making this model unsuitable for our study. Ovine, canine, and porcine models are more accurate models of the human adult trachea. The treatment of ATM with the TE-ETS developed in the present study does not require immunosuppression, can create an appropriate vascular network, preserves mucociliary clearance, and its implantation in the patient is technically simple.

The postoperative follow-up revealed fibrosis from 8 WPIs that increased by 12 WPIs and finally decreased by 16 WPIs. This fibrosis is considered the initial phase for regeneration of an ECM rich in collagen type I and type II and does not seem to produce tracheal stenosis; however, further studies have to be performed to better understand its significance. It was possible to determine early signs of tissue vascularization in the TE-ETS starting from 8 WPIs and until 16 WPIs.

Although we previously performed in vitro differentiation, it was uncertain if the microenvironment influenced cell phenotype. The histological analysis revealed the formation of chondrogenic nodules that showed cell organization and proteoglycan synthesis attributable to autologous oMSC-chondrocytes that were seeded during the implantation. This effect confirms that, in our model, the chondrogenic pre-differentiation of oMSC using TGF-β1 can be chondrogenically-primed oMSC and promote in vivo chondrogenesis. Other research groups using animal models have resected between 8 and 10 tracheal rings, producing cyanosis and stridor in the postoperative period. We decided to remove four rings to cause the airway collapse that defines tracheomalacia at the same time as ensuring that the animals presented with dyspnea and stridor only during exertion. This measure was considered humane, but it also represents a limitation of our study, since the sheep in our model had no severe clinical manifestations, as has been shown in other studies. It is important to recognize that this splint was designed for the management of tracheomalacia and not as a universal tracheal substitute. In tracheomalacia, the mucosal epithelial lining is healthy, and only the structural rigidity of the external framework is affected. The splint restores the lost structural rigidity of the airway, but it does not produce major luminal changes. Therefore, it is not indicated for clinical situations that require tracheal substitution, such as severe tracheal stenosis or extensive malignant neoplasms.
Conclusion

The TE-ETS developed prevents airway collapse and promotes chondrogenesis. Furthermore, it does not require exposure of the internal airway to the external environment, preserving mucociliary clearance. The TE-ETS fulfills several characteristics of the ideal stent: it is biocompatible, non-immunogenic, has an appropriate degradation rate, is easily available at a low cost, is easy to implant, and does not obstruct the airway in our ATM animal model.

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Conflict of Interests

The authors have no conflict of interests to declare.

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