Bridging the Gap: Using 3D Printed Polycaprolactone Implants to Reconstruct Circumferential Tracheal Defects in Rabbits

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Abstract

Introduction

The reconstruction of long segmental tracheal defects remains a surgical challenge. Researchers have investigated different types of polymers with or without biologic components to try to create a solution for this problem.

Objectives

1) To assess the feasibility of reconstructing 2 cm long circumferential tracheal defects with a 3D printed Polycaprolactone (PCL) implant in New Zealand rabbits. 2) To evaluate endoscopic, histologic and functional characteristics of a PCL tracheal implant over time.

Methods

A total of 10 New Zealand rabbits were included in the study with 2 rabbits per group that differed in time to sacrifice post-operatively. A model of a rabbit trachea was designed and 3D printed using a desktop printer. All rabbits underwent surgical excision of a 2 cm segment of cervical trachea which was reconstructed with a 2 cm 3D printed PCL implant. Rabbits were sacrificed at the following time points: 0,4,5,6, and 7 weeks post operatively. At these time points, a rigid bronchoscopy was performed and blinded evaluators calculated the percentage of airway stenosis. The tracheas were then harvested and prepared for histologic analysis.

Results

All rabbits survived to their date of sacrifice except for one. Rabbits were euthanized between 0-54 days post-operatively with a median of 30 days. All rabbits developed significant granulation tissue with an average percentage stenosis of 92.3±6.1%. On histological analysis, granulation was present with extensive neovascularization and mixed inflammatory cells. There was re-

epithelialization present on the luminal surface of the PCL implant near the anastomoses but absent at the center of the implant.

Conclusions

This study demonstrates that our 2 cm long 3D printed PCL tracheal implant can be used to reconstruct a tracheal defect of equivalent size in a New Zealand rabbit model in the short term. However, significant airway stenosis at the anastomotic sites occurs which will limit long term survival. Further research is warranted to limit the granulation tissue overgrowth.

Résumé

Introduction

La reconstruction de longs segments de défauts trachéaux demeure un défi chirurgical. Plusieurs études sur l'utilisation de différents types de polymères imprégnés avec ou sans facteurs biologiques comme substitut ont été réalisés par d'autres chercheurs.

Objectifs

1) Évaluer la possibilité de reconstruire des lacunes trachéales circonférentiels de 2 cm de long à l'aide d'un implant de polycaprolactone (PCL) imprimé en 3D chez des lapins de la Nouvelle-Zélande. 2) Évaluer les caractéristiques endoscopiques, histologiques et fonctionnelles d'un implant trachéal PCL pendant une durée de temps.

Méthodes

Un total de 10 lapins ont été inclus dans cette étude. Un modèle de trachée de lapin en PCL a été conçu et imprimé en 3D à l'aide d'une imprimante de bureau. Tous les lapins ont subi une excision chirurgicale d'un segment de 2 cm de la trachée cervicale, qui par la suite, a été reconstruite et remplacée avec un implant en PCL de 2 cm.

Les lapins ont été sacrifiés aux moments suivants: 0, 4, 5, 6 et 7 semaines post-opératoires. À ces moments, une bronchoscopie rigide a été réalisée et deux évaluateurs à l'aveugle ont calculé le pourcentage de sténose des voies respiratoires. Les trachées ont ensuite été récoltées et préparées pour une analyse histologique.

Résultats

Sur les 10 lapins, 9 ont survécu jusqu'à la date de leur sacrifice. Les lapins ont été euthanasiés entre 0 et 54 jours après l'opération, avec une médiane de 30 jours. Tous les lapins ont développé de la granulation sévère aux sites d'anastomoses avec un pourcentage moyen de sténose de 92.3 ± 6.1%. Lors de l'analyse histologique, la granulation était présente avec une néovascularisation étendue et des cellules inflammatoires mixtes. La ré-épithélialisation était présente sur la surface luminale de l'implant, proche des sites d'anastomoses mais était absente au centre de l'implant.

Conclusions

Cette étude démontre que notre implant trachéal PCL imprimé en 3D de 2 cm de long sans facteurs biologiques peut être utilisé à court terme pour reconstruire un défaut trachéal de taille équivalente dans un modèle de lapin. Cependant, une sténose importante des voies aériennes aux sites anastomotiques limitera la survie à long terme. Des études supplémentaires sont nécessaires pour limiter la prolifération des tissus de granulation.

Preface

Contribution of authors

Idea for project: Dr David S. Chan. Project supervisors: Dr. Sam J. Daniel and Dr. John J. Manoukian.

Chapters 1,2,5,6. Written by Dr David S. Chan

Chapter 3. Dr David S. Chan performed the scoping review and manuscript write up. Dr Naif Fnais and Imam Ibrahim helped as independent reviewers. Sam Daniel and John Manoukian supervised the review and approved the manuscript.

Chapter 4. Drs David S Chan, Sam J Daniel and John J. Manoukian participated in the conception and design of study. Dr Nathalie Gabra helped with the training process and supervised with the animal surgical procedures. Dr Ayesha Baig performed histopathology analysis following animal surgeries. Dr David Chan was the main author of the manuscript.

Disclosures

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Chapter 1: Introduction

1.1 Rationale

The trachea is a vital organ that allows air to move in and out of the lungs. It is composed of many cartilaginous rings and covered with ciliated respiratory epithelium on the luminal side and connective tissue, smooth muscle and vascular supply on the outer surface[1,2]. Congenital and acquired tracheal pathology can cause significant symptoms, death and sometimes surgery may be necessary to remove the diseased trachea. In cases of small segmental resections, end-to-end anastomosis is possible as a repair option; however, in cases where there is a long segment involved, more complex options with potentially fatal risks may be required[1].

Trends in the literature involve embedding different 3D printed scaffolds with a cellular component. For example, Jang et al. 2014 used a scaffold with PCL and collagen nanofibers with human umbilical cord serum to facilitate epithelial cell migration in a tracheal patch in rats[3]. In a study by Gao et al 2017, circumferential tracheal reconstruction in rabbits was performed using a PCL graft embedded with chondrocytes. Over 80% of their rabbits had significant granulation tissue formation, their luminal surface lacked epithelial tissue, and there was minimal cartilage growth which they suspected was due to the inflammatory response[4].

Though stem cell technology in medicine appears to have significant potential, it is important to note its risks. These include poor long-term survival of stem cells, immune rejection of donor cells, and the potential to become oncogenic due to their unlimited capacity to replicate. The use of stem cells has raised severe ethical and social concerns and their use comes at a high cost requiring specialized equipment and personnel.[5]

With the advancements of 3D printing in medicine, a possible solution to the long circumferential tracheal defects is seen. 3D printing offers a customizable solution that can possibly allow for the gap to be bridged with a printed polymer that replaces the missing tracheal segment. Few studies have been undertaken in order to find suitable materials [3,6-8]. Preliminary research at our center demonstrated the feasibility of using a 3D printed Polycaprolactone (PCL) matrix to replace 1-cm long, segmental tracheal defects in 2 rabbits. The rabbits survived the length of the study (6 weeks) and there was epithelium and cartilage growth within the matrix.

Despite showing promise, there are very few studies that have evaluated the use of PCL in repair of long segment circumferential defects. As a result, this thesis set out to explore these issues, leading to the thesis objectives listed in the following section. This thesis will add to the literature of tracheal reconstruction and will have merit after comparison with existing studies in this area.

1.2. Objectives

The main objective of this thesis was to create and assess the feasibility of using a 3D printed PCL implant to reconstruct long segment circumferential tracheal defects in an animal model. The second objective was to perform comprehensive literature reviews of tracheal pathologies, and of the use of PCL in tracheal surgeries.

1.3 Thesis outline

Chapter 2 presents a review of tracheal anatomy, tracheal stenosis and tracheomalacia. Chapter 3 presents a scoping review of the use of PCL in tracheal surgeries in vivo (manuscript 1). Chapter 4 is a study of the use of a 3D printed 2-cm PCL implant for the reconstruction of circumferential tracheal defects of equivalent size (manuscript 2). Chapter 5 discusses future

directions. Chapter 6 presents overall conclusions and lists claims of originality. Chapter 7 contains the bibliography. Chapter 8 lists abbreviations. Appendices are at the end of the thesis.

Chapter 2: Tracheal Pathologies

2.1 Anatomy of the trachea

The trachea is a cartilaginous tube that extends from the larynx superiorly to the main bronchi inferiorly, and acts as a conduit between the outside atmosphere and the lung parenchyma. It transmits oxygen to the lung during inspiration and expels carbon dioxide during expiration [9]. The carina is a cartilaginous ridge that marks the end of trachea, where it bifurcates to form the two main bronchi, with the right bronchus having a steep-angled take off, compared to the more horizontal left bronchus take-off. Approximately 18 to 22 cartilaginous rings form the tracheal framework which is connected posteriorly by a membranous wall made up of the longitudinal trachealis muscle. Tracheal diameters vary with age and sex. At birth to 1 month, the average length is 3.8 cm, the average anteroposterior diameter is 5.7 mm, and the average transverse diameter is 6 mm. These values continue to increase to reach the adult values, which are 9-11 cm length, 13-19 mm anterior-posterior diameter, and 19-20 mm transverse diameter [10]. The cross-sectional shape of the tracheal lumen changes in shape with age. At birth it is circular and as the child grows the lumen usually tends to become more ovoid. The mucosa that covers the tracheal lumen is lined by ciliated pseudostratified columnar epithelium containing mucous producing goblet cells. The blood supply to the trachea arises from the right and left thyrocervical trunks, and the tracheoesophageal branches of the inferior thyroid arteries.[9]

The trachea sits anteriorly in the neck and back in the mediastinum and is surrounded by several vital structures and vessels. The isthmus of the thyroid gland connects its two lobes at the level of the 2nd or 3rd tracheal ring, while the two lobes of the thyroid sit anterolateral to the cervical trachea. The esophagus is directly posteriorly related to the trachea along its course, where

fibroelastic membranes and some muscle fibers connect the trachealis muscle and the longitudinal muscle of the outer esophagus. The right and left recurrent laryngeal nerves are the vagal branches that innervate the true vocal cords which run along the trachea. Several large vessels that come in direct contact with the trachea including the brachiocephalic artery, the carotid arteries, the pulmonary truck with its right and left branches, and veins such as the superior vena cava, the jugular veins and the azygous vein.[9]

2.2 Tracheal stenosis

The narrowing of the trachea is known as tracheal stenosis. It is a serious condition that can interfere with normal breathing and even death if not managed appropriately. Tracheal stenosis is commonly iatrogenic, following prolonged endotracheal intubation or tracheostomy. However, other causes can also result in tracheal stenosis, such as inflammation, trauma, tumors, infection, or autoimmune disorders. Tracheal stenosis can also develop as a side effect of radiation therapy that has been used to treat head or neck tumors. In rare cases, infants are born with congenital tracheal anatomical abnormalities leading to tracheal stenosis.[11]

Depending on the etiology, symptoms of tracheal stenosis include shortness of breath, stridor, cough, hemoptysis, recurrent upper respiratory tract infections, etc. The diagnosis of tracheal stenosis can be made by direct visualization by rigid or flexible bronchoscopy, and through imaging modalities such as CT scans or MRIs. Treating tracheal stenosis can be extremely challenging. Currently, the available treatment options include endoscopic surgery for mild to moderate cases of stenosis, where narrowed areas can be widened using balloons, excised or debrided. Tracheal/cricotracheal resection is an open surgical option where the stenosed trachea is resected. In a short defect, primary anastomosis is possible with or without suprahyoid

and/or tracheal release. Segments longer than 2 cm in children or 5 cm in teenager/adults are difficult to manage because the gap is too long and because of the of excess tension that is generated at the anastomotic sites with primary closure [4]. Currently, the slide tracheoplasty is a common option for longer segmental defects, where the tracheal incisions are made are at an angle to create beveled shaped ends. It is then slid up on itself and reconnected to form a shorter but wider trachea[12].

If surgery fails or is not possible, a tracheobronchial airway stent can also be placed at the narrow part to maintain an airway. Stents can be metal, silicone, or hybrid tubes, and are used for both short- and long-term treatments for tracheal stenosis[13]. Mediastinal tracheostomies may sometimes be necessary to by-pass the level of stenosis[1].

Despite previously-used innovative surgical techniques, long segment tracheal stenosis remains a challenge for surgeons, and subjects patients to high operative and anesthetic risks. Recently, investigators have researched usage of a combination of different synthetic polymers and biologic materials to create a tracheal replacement to reconstruct large segment tracheal defects.

2.3 Tracheomalacia

Tracheomalacia, which directly translates to ‘soft trachea’, is a condition that leads to excessive tracheal collapsibility with respiration. This is often related to laxity of the posterior tracheal wall or weak cartilage. Tracheomalacia may be localized to a small segment of trachea or generalized throughout and can vary in severity. Tracheomalacia can also be congenital or acquired. The primary causes of tracheomalacia can be associated with congenital anomalies of the aerodigestive tract (ie tracheoesophageal fistulas), genetic factors, prematurity and multiple

congenital syndromes including: Trisomy 21, CHARGE, Opitz, DiGeorge, Crouzon, etc. On the other hand, acquired tracheomalacia can be related to external compression of the trachea from vascular abnormalities, skeletal anomalies (ie. Scoliosis, pectus excavatum), infection, tracheobronchial injury, iatrogenic causes (ie. prolonged intubation, tracheostomy), as well as tumors.[14]

Common symptoms of tracheomalacia include a brassy/barking cough, stridor, wheezing, recurrent and prolonged respiratory infections, dying spells, feeding difficulties and dyspnea. Investigations for tracheomalacia must include a dynamic study to be able to assess collapsibility of the trachea with respiration. Imaging modalities include fluoroscopy, dynamic MRI, and multi-detector CT; however, the gold standard is direct visualization with flexible bronchoscopy. Rigid bronchoscopy can play a role, but it may splint the airway open and therefore may be less reliable in detecting tracheomalacia. The surgical options for tracheomalacia aim at maintaining a patent airway that does not collapse with respiration. Aortopexy, tracheopexy, internal stents, external splints can all be attempted to achieve this goal. In other cases, segments of tracheas can be resected and reconstructed. [14]

2.4. Linking statement

The following chapter, Chapter 3, presents a scoping review of the uses of PCL in tracheal surgeries in-vivo in animal studies as well as human cases. This will help determine what has been investigated and identify the gaps of knowledge on this topic.

Chapter 3: Exploring Polycaprolactone in Tracheal Surgery: A Scoping Review of in-vivo studies (Manuscript 1)

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3.1. Abstract

Introduction Tracheal pathology can be life-threatening if not managed appropriately. There are still some surgical limitations today for certain pathologies, such as in severe tracheomalacia, or when long segments of trachea need to be resected. Poly(ε-caprolactone) (PCL) is a polymer that has recently gained popularity for its use in tracheal surgeries in animal models and in certain human pediatric cases in hopes of addressing these difficult situations. PCL can be 3D printed or manufactured through molds to create tracheal stents, splints, patches and even to reconstruct full circumferential tracheal defects.

Objective To perform a scoping review, and explore insights into the applications of PCL for tracheal surgeries in-vivo.

Methods A literature search in Embase, MEDLINE, and BIOSIS was performed to include all articles available prior to December 21, 2018 without any language restrictions. We included all original research that investigated the use of a PCL implant, stent, splint, scaffold, or graft in tracheal surgeries in-vivo. Assessment of all articles were performed by two independent authors prior to inclusion for analysis.

Results A total of 27 articles were included in the study. All articles were original research studies, primarily consisting of interventional studies (92.4%), there was also 2 case reports (7.4%). Articles were published in the last decade, publications range from 2009-2019. The most common animal model used for the tracheal surgeries were the New Zealand rabbits (n=19, 70%). Two studies (7%) also described the use PCL in a total of 4 human cases. To investigate the PCL reconstructed airways, histology and bronchoscopy were the most commonly implemented methods of analysis in 88.9% and 70.4% respectively. Airway analysis was also

done using imaging modalities including CT scan (n=9, 33.3%), MRI (n=2, 7.4%), X-ray (n=1, 3.7%). 17 (62.9%) of the studies used 3D printing processes to create their PCL implants.

Conclusions Overall, this review demonstrates the feasibility of PCL in tracheal reconstruction and tracheal stenting/splinting. It highlights common trends and the limitations of the literature thus far on this topic.

Key Words: Poly(ε-caprolactone) (PCL), Trachea, Airway Reconstruction, 3D printing

Abbreviations: NZR (New Zealand Rabbits), BMSC (Bone marrow stem cells), HTMSC (human turbinate mesenchymal stromal cells), ECM (extracellular matrix), Mesenchymal Stem cells (MSC)

3.2. Introduction

With advancements in tissue engineering and 3D printing, many studies have combined the two in hopes of improving the technology that are currently available for tracheal surgeries. Tissue engineering in combination with 3D printing allows for customizable, targeted development of scaffolds, stents, splints and options for tracheal replacements in the context of treatment of tracheomalacia and for the reconstruction of tracheal defects. [4]

Tracheomalacia is defined as a soft or weak cartilaginous support of the trachea which can lead to the collapse and narrowing of the airway lumen during respiration. Tracheomalacia is the most common congenital disease of the trachea and is more commonly seen in premature infants. It can also be acquired and occur secondarily from infection, prolonged intubation, tracheostomy, trauma, tumors, and from external compression by vascular structures. In mild to moderate tracheomalacia, observation or continuous positive airway pressure therapy may be effective; however, if these fail and the malacia is severe, surgery is usually indicated. Aortopexy can be performed if there is a suspected component of vascular compression contributing to the tracheomalacia. Otherwise, external tracheal splints or internal tracheal stents are used to maintain the airway lumen[15].

Open surgical procedures such as tracheal segmental resection and reconstruction can be used in the management of tracheomalacia but is most commonly reserved for cases of tracheal stenosis, tracheoesophageal fistulas or for malignancy. In a short defect, primary anastomosis is possible with or without a suprahyoid release and currently, conventional options for longer segment tracheal defects include pericardial patch, tracheal autograft, and slide tracheoplasty. Despite the innovative surgical techniques, long segment tracheal stenosis remains a challenge for surgeons, and subjects the patients to high operative and anesthetic risks. Segments longer

than 2 cm in children or 5 cm in teenager/adults are difficult to manage because the gap is too long and because of the of excess tension that is generated at the anastomotic sites with primary closure [4].

Poly(ε-caprolactone) (PCL) is an ideal material that has gained popularity for its use in tracheal tissue engineering. It has strong mechanical characteristics to maintain the airway, non-toxic degradation products and good biocompatibility allowing for cellular growth within the PCL implant[16]. PCL is widely available, can be used in blends with other polymers and has a low melting temperature (59-64^{o}C) allowing for it to be easily manufactured [17,18].

The objective of this scoping review is to systematically review the literature to explore the applications of PCL for tracheal surgeries in-vivo.

3.3. Methods

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The Preferred Reporting Items for Systematic Reviews and Meta-analyses reporting guideline Extension for Scoping Reviews was followed for this scoping review (PRISMA-ScR) [19].

3.3.1. Search strategy

Relevant articles were identified by searching the following databases on December 21, 2018, with no publication date restrictions: Ovid MEDLINE, Ovid EMBAS and Clarivate BIOSIS. There were no restrictions based on the language of published article. Combinations of the following search terms were used: *trachea, three dimensional printing, polycaprolactone*.

The search strategy was developed by a medical research librarian at the McGill University Health Center (See Supplement 1).

Supplement 1. Scoping Review Search Strategy

Medline [Ovid] (December 21, 2018)
Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily <1946 to Present>

#	Searches	Results
1	Trachea/	35606
2	trachea*.tw,kf.	60771
3	1 or 2	72962
4	exp Printing, Three-Dimensional/	2726
5	(((3-d or 3d or 3-dimension* or three-dimension* or 3dimension*) adj4 (print* or bioprint* or scaffold* or graft*)) or stereolithograph*).tw,kf.	13009
6	(PCL or polycaprolacton* or (poly adj5 caprolacton*)).tw,kf.	12578
7	4 or 5 or 6	25296
8	3 and 7	139

Embase [Ovid] (December 21, 2018)
Embase <1974 to 2018 December 20>

#	Searches	Results
1	exp trachea/	33636
2	trachea*.tw,kw.	73163
3	1 or 2	82522
4	exp three dimensional printing/	5919
5	(((3-d or 3d or 3-dimension* or three-dimension* or 3dimension*) adj4 (print* or scaffold* or graft*)) or stereolithograph*).tw,kw.	15479
6	polycaprolactone/	8453
7	(PCL or polycaprolacton* or (poly adj5 caprolacton*)).tw,kw.	15654
8	4 or 5 or 6 or 7	33057
9	3 and 8	247

Biosis [Clarivate] (December 21, 2018)
Indexes=BCI Timespan=All years

#	Searches	Results
1	TS=(trachea*)	52,578
2	TS=(((3-d or 3d or 3-dimension* or three-dimension* or 3dimension*) NEAR/4 (print* or bioprint* or scaffold* or graft*)) or stereolithograph*)	8,643
3	TS=(PCL or polycaprolacton* or (poly NEAR/5 caprolacton*))	9,608
4	#3 OR #2	17,659
5	#4 AND #1	71

3.3.2. Article selection

We selected all original research articles that evaluated the use of PCL in-vivo for tracheal surgery. We included animal and human studies but only those in which PCL was directly used to reconstruct, support or stent the trachea in-vivo. Articles where PCL was combined with another material, and/or biologic or cellular component were also included.

3.3.3. Article review

Our review process involved two stages. In stage 1, two of the authors (D.S.C. and N.F.) screened the titles and abstracts of each article independently. If there were any disagreements between the reviewers, there was a discussion amongst them. If at that point, there was still a disagreement, the article was included for stage 2. In the second stage, the complete manuscript of the selected studies were independently reviewed by the two same authors. If there were disagreements between them for a given article, a 3rd author (I.I.) arbitrated for further inclusion. There was no blinding to authors, affiliations or publishing journal.

Extracted information included type of species studied, number of subjects, PCL implant construct design, type of additive materials, method of analysis and time points. Studies were

categorized by the type of PCL construct implant design into 1) Circumferential Reconstruction, 2) Partial Reconstruction, 3) Stent/Splint.

3.3.4. Statistical Analysis

Since the studies were heterogeneous in terms of outcomes and endpoints, data was not pooled across studies. Analysis was mainly descriptive, we summarized data as counts and means.

3.4. Results

Our initial search yielded 457 articles, 186 duplications were automatically removed which left us with a total of 271 articles for review. After the first screening stage of titles and abstracts, 69 articles were selected for full text review. After the second stage, 27 articles were included for the final analysis, all were complete manuscripts. The majority of articles were excluded because they were not related to the topic or because they were not in-vivo studies of PCL use in tracheal surgery (See Supplement 2). All identified articles for the final review were written in English.

11 studies were classified as circumferential tracheal reconstruction, 11 as partial tracheal reconstruction, and 5 as tracheal stents/splints. Categorizing the studies was done to better compare similar types of interventions. All articles were original research studies, primarily consisting of interventional studies (92.4%), there was also 2 case reports (7.4%). All articles were published in the last decade; publications range from 2009-2019. The most common animal model used for the tracheal surgeries were the New Zealand rabbits (n=19, 70%). Two studies (7%) also described the use PCL in a total of 4 human cases. To investigate the PCL reconstructed airways, histology and bronchoscopy were the most commonly implemented

methods of analysis in 88.9% and 70.4% respectively. Airway analysis was also done using imaging modalities including CT scan (n=9, 33.3%), MRI (n=2, 7.4%), X-ray (n=1, 3.7%). 17 (62.9%) of the studies used 3D printing processes to create their PCL implants. (Tables 1)

Supplement 2. PRISMA flow diagram

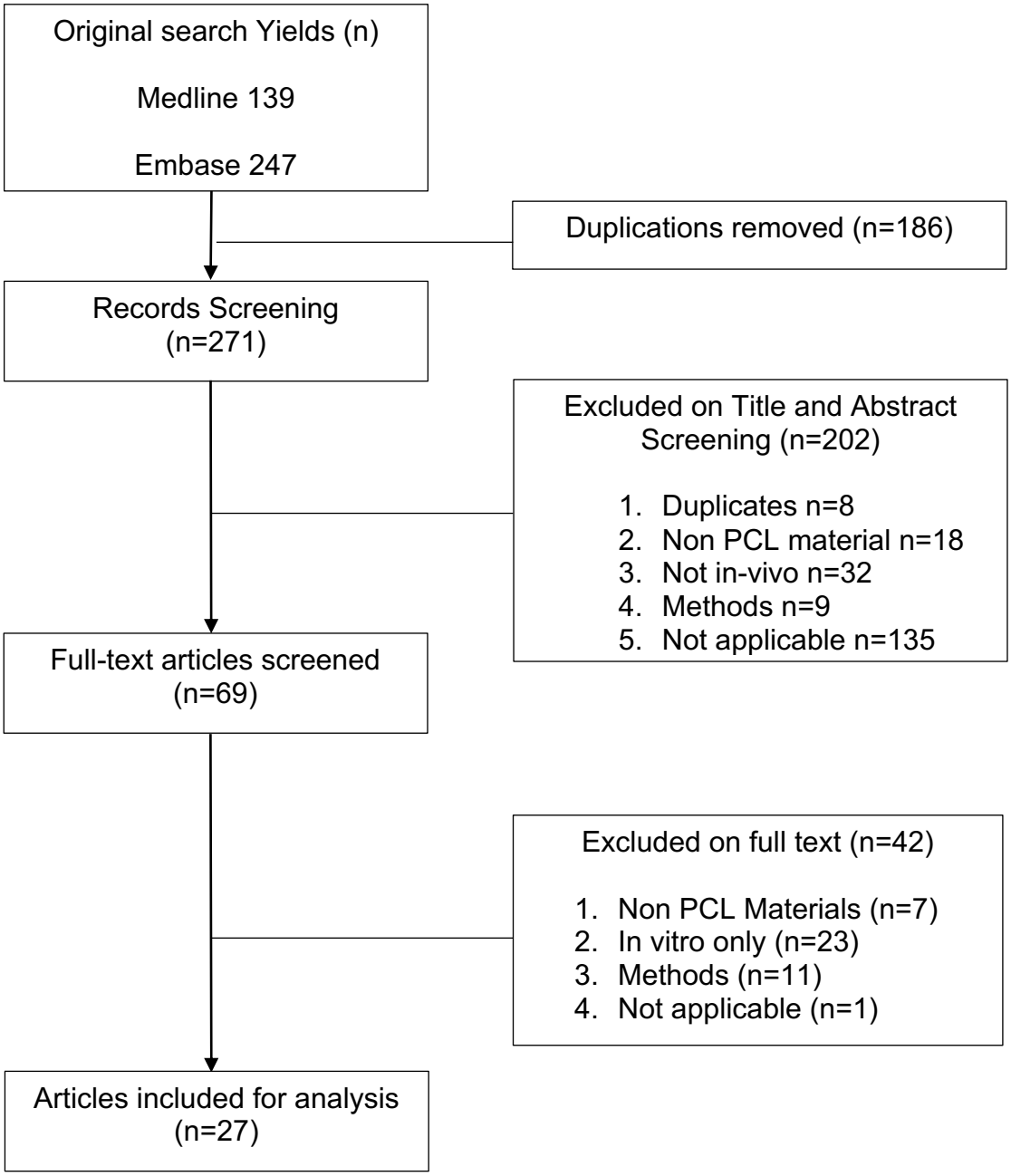

Table 1. Common findings Between Articles

	No. (%) of 27 articles
Country of Origin	
Korea	13 (48.1)
United States of America	6 (22.2)
China	2 (7.4)
Taiwan	5 (18.5)
Japan	1 (3.7)
Species	
Rabbit	19 (70.4)
Pig	4 (14.8)
Human	2 (7.4)
Sheep	1 (3.7)
Rat	1 (3.7)
Implant design/testing	
3D printed	17 (62.9)
Scanning Electron Microscopy	12 (44.4)
Mechanical testing	8 (29.6)
In-vivo or post mortem analysis	
Bronchoscopy	20 (74.0)
Histology	24 (88.9)
Immunohistochemistry	5 (18.5)
CT scan	10 (37.0)
X-ray	1 (3.7)
MRI	2 (7.4)

3.5. Discussion

To our knowledge, this is the first review conducted to comprehensively identify the uses and outcomes of PCL in tracheal surgeries in-vivo. PCL's robust mechanical properties and good biocompatibility has led it to become increasingly popular for the use of tracheal reconstruction in the last decade in animal models. PCL can now be 3D printed and modeled to any shape or size which can conveniently be used with airway reconstruction. It can also be combined with

other types of polymers to utilize characteristics of different materials[20]. Because of its relatively low melting point, PCL is easily molded and can even be used with commercial desktop 3D printers as demonstrated by Kaye et al.[21]

In this review, overall, the most commonly used animal model is the New Zealand Rabbit as authors argue that it is the ideal model for tracheal surgery. Rabbits have a long cervical trachea that is easily surgically accessible, their trachea closely resembles a human trachea in structure and its size is similar to that of a human infant. Rabbits have a more diverse genetic background compared to rodents which makes the model a better overall approximation to human's genetic diversity. They are widely available, simple to manage and cost-effective for interventional studies [22,23]. However, sheep, pig, and dog models may be better to replicate the size of older teenage children with adult sized tracheas but these large animals are costly to house and manage post-operatively [20,24-27].

Within our review, we identified 3 broad categories for the use of PCL in tracheal surgeries 1) Circumferential Reconstruction, 2) Non-circumferential Reconstruction, and 3) Stents/Splints to better compare characteristics and outcomes of the studies. We analyzed them in the above groups as it would be difficult to compare the outcomes of these groups altogether, as the surgical implications between the 3 are quite unique.

Circumferential defects are the most difficult to reconstruct, with longer defects being more challenging than shorter ones. Longer defects are to be more likely to induce granulation tissue causing stenosis and respiratory difficulties. This is likely due to the disruption of the mucosa and ciliary clearance as re-epithelization of the implanted segment will undoubtedly take more time in longer implants. Overall, circumferential stenosis from granulation at the

anastomotic sites, was mentioned in almost all studies with circumferential reconstruction. Different cellular and biologic components were added to the PCL construct in many of these studies to potentially accelerate cellular growth and migration and diminish granulation formation. However, granulation and stenosis occurred regardless of what additive was added to the PCL construct. This is the main challenge that needs to be addressed in circumferential PCL grafts, as the longest life-spans of the subjects in the literature range from 34-81 days (median 56 days), when excluding the study by Tsukada et al which is a clear outlier [20]. Most of these were based on New Zealand rabbit models with tracheal lumens of 4.7-5.9 mm, whereby millimetres of narrowing will significantly increase airway resistance as per Poiseuille's Law, and could therefore be life-threatening [28]. Tsukada et al compared different combinations of Polyglactin, PCL, and Poly-L-Lactide to create 5 cm circumferential acellular tracheal implants in dogs. They demonstrated the longest survival compared to any other study when combining all three materials with animals living up to 2 years and some still alive at the end of the study.[20] Lee et al. evaluated the use of immunosuppressive therapy after tracheal replacement though, they concluded there were no beneficial effects[29]. At the time being, there does not appear to be adequate options for circumferential tracheal reconstruction in the animal model because of the high likelihood of stenosis and granulation tissue that form at the anastomotic site or at the center of the implant. Future studies may want to focus on its prevention or methods of addressing the stenosis for example, balloon tracheoplasty, stenting, corticosteroid injection, or different combinations of immunosuppressive and anti-inflammatory medications. (Table 2)

Table 2. Summary of Circumferential Reconstruction Articles (n=11)								
Author	**Year**	**Subjects**	**n**	**Additive to PCL implant**	**Size (mm)**	**Curvature (°)**	**3D Printed**	**Longest Survival (days)**
Ahn[30]	2017	NZR*	12	None	20	360	x	56
Bhora[27]	2017	Pigs	5	ECM*/Collagen layer	15	360 vs 270	x	34
Gao[4]	2017	NZR	21	Chondrocytes then implanted in subcutaneous tissue of nude mice	16.5	360	x	70
Lee[31]	2015	NZR	6	1) Pluronic F127 membrane 2) PCL alone	20	360	x	46
Lee[32]	2017	NZR	24	1) Collagen layer/HTMSC* 2) Collagen layer/HTMSC + cyclosporine 3) Collagen layer/HTMSC + azithioprine 4) Collagen layer/HTMSC +azithioprine/cyclosporine	12	360		28
Lin[33]	2009	NZR	6	Chondrocytes	10	360		81
Lin[34]	2011	NZR	6	Genipin	10	360		51
Park HS [35]	2018	NZR	10	1) Implanted in omentum 2) PCL alone	15	360	x	56
Park JH[36]	2018	NZR	20	1) Collagen layer/HTMSC 2) ECM hydrogel/HTMSC	10	360	x	56
Tsao[16]	2014	NZR	12	1) Chondrocytes 2) BMSC* 3) BMSC/Chondrocytes 4) PCL alone	10	360		-
Tsukada[20]	2009	Dogs	17	1) Polyglactin 2) L-lactide/PCL copolymer + Polyglactin 3) L-lactide/PCL copolymer + Poly-L-lactide	50	360		730 - Ongoing

In the non-circumferential group, different shapes and sizes were evaluated, from small rectangular anterior tracheal defects to 20 mm long 270° tracheal reconstruction. In this group, it appears granulation and stenosis is less of an issue. Park et al reconstructed a 7x10 mm defect with a bare PCL implant with no evidence of stenosis and minimal granulation tissue at 8 weeks on bronchoscopy. On histology, regeneration of ciliated epithelium and neovascularization on the luminal surface of the graft was noted [37]. Similar results were seen in many of the studies

with smaller rectangular shaped defects [3,22,37,38]. When larger defects were created and repaired on larger animals, results were variable. Rehmani et al reconstrued a 40 x 16 mm defect in 7 pigs with a PCL implant covered with an extracellular matrix and found that 5/7 pigs at 3 months had adequately sized tracheal lumen with minimal granulation tissue and stenosis[26]. However, in the study by Townsend et al, all sheep subjects were euthanized prior to the end of the study because of respiratory distress secondary to tracheal narrowing at the level of the reconstruction with a 15x25 mm PCL implant [25]. (Table 3)

Table 3. Summary of Non Circumferential Reconstruction Articles (n=11)

Author	Year	Subjects	n	Additive to PCL implant	Size (mm)	Curvature (°)	3D Printed	Longest Survival (days)
Bae[39]	2018	NZR*	12	1) Epithelial cells/BMSC* 2) Epithelial cells/chondrogenic-differentiated BMSC	10x10	180	x	84
Chang[40]	2014	NZR	4	Fibrin/MSC*	10x10	180	x	56
Jang[3]	2014	Rat	14	1) Collagen 2) Collagen/human umbilical cord serum	4x8			49
Joo[41]	2014	NZR	8	None	5x10			56
Kaye[21]	2019	NZR	7	Hyaline chondrocytes	20	270	x	42
Kwon[38]	2013	NZR	15	Pluronic F127 membrane	3 ring square	120		84
Park HS[22]	2018	NZR	4	None	7×10		x	56
Park JH[37]	2012	NZR	8	Collagen	5x10		x	56
Park JH[42]	2015	NZR	12	1) Collagen 2) Collagen/HTMSC*	15	120	x	28
Rehmani[26]	2017	Pig	7	ECM*	40x16	180	x	90
Townsend[25]	2018	Sheep	5	None	15x25		x	70

The PCL stents and splints in 4/5 studies (80%) were designed for the management of tracheomalacia [24,43-46]. In this category, the use of PCL has already moved from animal studies to use in human patients. Two of the studies reported the implantation of 3D printed PCL constructs in human patients with seemingly good results. Huang et al. trialed a 3D printed external splint in a 46 y.o. female with tracheomalacia secondary to tracheal tuberculosis who report clinical improvement in respiratory symptoms although follow up was only documented after 3 months

[45]. Morrison et al. describe the use of 3D printed PCL splints in 3 infants with terminal tracheobronchomalacia who had immediate and sustained improvement in their clinical status, with follow ups from 11 to 38 months [43]. PCL implants can also be created to have drug eluting properties; for example Chao et al developed a cisplatin eluting PCL tracheal stent for the management of endotracheal local chemotherapy to treat malignant airway obstruction [46]. (Table 4)

Table 4. Summary of Stent/Splint Articles (n=5)

Author	Year	Subjects	n	Implant Type/Additive	Size (mm)	Curvature	3D Printed	Longest Timepoint (days)
Chao[46]	2013	NZR*	15	Stent + Cisplatin		360		35
Huang[45]	2016	Human	1	Splint	70	270	x	3 months - ongoing
Liu[44]	2011	NZR	6	Stent	22	360		231
Morrison[43]	2014	Human	3	Splint	11 - 23	270	x	38 months - ongoing
Zopf[24]	2014	Pig	6	Splint		270	x	6

After conducting this review, we have identified some gaps within the literature. The majority of studies in animals have endpoints less than 90 days; in fact, only 2 animal studies goes beyond this time point [20,44]. Though costly, it is imperative to explore long term effects of the PCL implants especially prior to their use in human tracheal wall reconstruction. Also, many studies do not use a control as part of their methods; often a cellular or biologic component is added to the PCL scaffold without comparing it against a bare PCL scaffold, with only 3 studies doing so [16,31,35]. Another three studies evaluated a PCL scaffold without any additives, and these showed similar survival outcomes as the other studies. Ahn et al investigated PCL scaffold alone for circumferential reconstruction of a long segment (2cm) in rabbits and had survival endpoints up to 8 weeks which is comparable to studies with added components to the PCL implant [30]. Similarly, partial tracheal reconstruction with a PCL scaffold alone was performed in 3 studies

with survival endpoints up to 56 days, 56 days and 70 days [22,25,41]. Many of the studies evaluated the addition of different types of cells within the PCL scaffold including chondrocytes, human turbinate mesenchymal stem cells and bone marrow derived stem cells, in hopes of accelerating cartilage and epithelial growth within the PCL implant. However, the process and benefit of using stem cells in tracheal reconstruction in-vivo still remains unclear and some authors believe that using cellular components may actually cause more of an inflammatory reaction, which may lead to granulation tissue and stenosis [25]. If cellular or biologic components are added to a PCL implant, they should ideally be compared to a control to truly assess how they compare in terms of survival, granulation tissue, and integration.

Based on our review of the current literature, we did not find a standardized method to analyze the integration of the PCL tracheal implants in in-vivo models. Most studies included bronchoscopy and histology to examine the tracheal lumen patency and cellular growth, where various histologic and immunohistochemistry stains were used. Some studies used imaging modalities including MRI, CT and X-Ray to examine the tracheal lumen for stenosis. Survival was documented in all studies which in our opinion is the most important outcome that will determine if PCL can further be implemented in human cases.

3.5.1 Limitations

Inherently, a scoping review provides less depth of information compared to a systematic review or a meta-analysis which is a limitation of our study. However, we believe that this method was the most appropriate for this emerging area of research with a only small number of studies that are heterogenous in nature. We performed the literature search in 3 different large

databases, however it is always possible that some articles that may actually be related to our area of research were missed and therefore not included in our manuscript.

3.6. Conclusion

PCL is a promising 3D printed biodegradable material that can be used for tracheal surgery in pediatrics. Overall, this review showed the feasibility of PCL grafts for tracheal replacement, current uses of PCL stents and splints in human patients, and the limitations of the literature thus far on this topic. We hope that this review will prompt researchers to fill some of these gaps in the near future of this promising area of research.

3.7. Linking statement

This review suggests that the main gaps in the literature are the limited number of studies evaluating: 1) PCL as an option to reconstruct long segmental defects in animal models, (2) PCL as a bare implant without additives. The following chapter (Chapter 4, Manuscript 2), presents an experimental study using rabbits that aimed to help fill this gap.

Chapter 4: Bridging the Gap: Using 3D Printed Polycaprolactone Implants to Reconstruct Circumferential Tracheal Defects in Rabbits (Manuscript 2)

Running Title: 3D Printed PCL in Tracheal Reconstruction

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4.1. Abstract

Objective: 1) To assess the feasibility of reconstructing 2 cm long circumferential tracheal defects with a 3D printed Polycaprolactone (PCL) implant in rabbits. 2) To evaluate endoscopic, histologic and functional characteristics of a PCL tracheal implant over time.

Methods: 10 New Zealand rabbits were included in this study. A 2 cm 3D printed PCL tracheal implant was created. All rabbits underwent surgical excision of a 2 cm segment of cervical trachea which was reconstructed with the implant. Rabbits were sacrificed at the following time points: 0,4,5,6, and 7 weeks post operatively. At these time points, a rigid bronchoscopy was performed, and blinded evaluators calculated the percentage of airway stenosis. The tracheas were then harvested and prepared for histologic analysis.

Results: All rabbits survived to their date of sacrifice except for one. Rabbits were euthanized between 0-54 days post-operatively with a median of 30 days. All rabbits developed significant granulation tissue with an average percentage stenosis of 92.3±6.1%. On histology, granulation was present with extensive neovascularization and mixed inflammatory cells. There was re-epithelialization present on the luminal surface of the PCL implant near the anastomoses but absent at the center of the implant.

Conclusions: This study demonstrates that our 2 cm long 3D printed PCL tracheal implant can be used to reconstruct a tracheal defect of equivalent size in a New Zealand rabbit model in the short term. However, significant granulation tissue formation limits long term survival. Further research is warranted to limit the granulation tissue overgrowth.

Level of Evidence: 2

Key Words: PCL (polycaprolactone), tracheal reconstruction, 3D-printed

4.2. Introduction

The trachea is a vital organ that can be affected by various medical conditions. Congenital and acquired tracheal pathology can cause significant symptoms and even death. Conditions include tracheomalacia, tracheal stenosis from prolonged intubation, autoimmune conditions, and cancers that arise from or invade the trachea. Occasionally, segmental tracheal resection may be necessary as part of the treatment plan. Surgical options to repair the trachea following a segmental resection can be very challenging and subject patients to high operative risks. In a short defect, primary anastomosis is possible with or without a suprahyoid release. Currently, a slide tracheoplasty which carries a mortality rate ranging between 5-30%[12,47] is the conventional option for tracheal stenosis and longer segment tracheal defects. Other options when such techniques have failed or are not feasible, include tracheal stents or mediastinal tracheostomies[1]. Interestingly, Delaere and the Leuven Tracheal Transplant Group have recently described 6 successful cases of tracheal allotransplantation in human patients for the use of long segment defects. Whereby a trachea from a deceased donor was placed and wrapped in the recipient's forearm fascia. After several months, an orthotopic transplantation is performed of the trachea with a vascularized forearm free flap. [48,49]

Multiple authors have also investigated different combinations of polymers, stem cells, and growth factors in hopes of designing a tracheal substitute to reconstruct tracheal defects in animal models.. Because of its favorable characteristics, polycaprolactone (PCL) is one of the polymers that has gained popularity for its evaluation in airway reconstruction in animal models. It has strong tensile properties, a low melting point, and allows for cellular growth along its surface. PCL can also easily be 3D printable which allows for a customizable solution to reconstruct tracheal defects[50]. PCL has also successfully been used in select human pediatric cases as external tracheal

splints for severe tracheomalacia[51,52].

The use of stem cells has gained popularity in organ regeneration in the last few decades, the trachea included. PCL has been embedded with different types of stem cells including epithelial cells, bone marrow stem cells, chondrocytes, human turbinate mesenchymal stem cells, etc[50]. Tracheal defects reconstructed with PCL have ranged in size from partial wall defects to full circumferential (360^{o}) defects in animal models. Partial reconstructed defects using PCL, regardless of the cellular additive, seem to have good results with appropriate cellular regeneration and minimal granulation tissue formation at the level of the implant. On the other hand, complete circumferential defects reconstructed with PCL in the majority of cases were limited by the significant granulation tissue formation especially with longer PCL implants regardless if stem cells were used.[50] Interestingly, few studies have compared PCL implants with biologic additives to bare PCL implants in the reconstruction of circumferential tracheal defects in animal models. Also, the true clinical value for stem cells applied to a non-vascularized scaffold is unknown[53]. We believe it is imperative to evaluate the baseline reaction to the bare PCL implant to be able to adequately assess adding biologic factors to it or as using it as a scaffold for a vascularized flap.

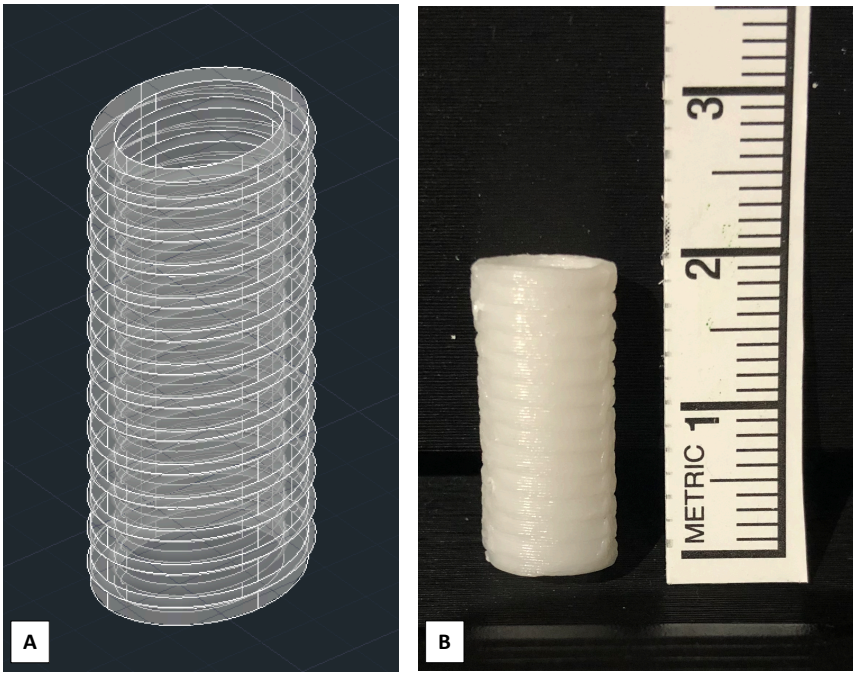
Thus, the objectives of this study are 2-fold: 1) To assess the feasibility of reconstructing 2 cm long circumferential tracheal defects with a 3D printed PCL implant in New Zealand rabbits. 2) To evaluate endoscopic, histologic and functional characteristics of a PCL tracheal implant over time.

4.3. Methods

4.3.1. Implant

A 3D model was designed with the Autodesk AutoCAD software® (Figure 1A) and then 3D printed (Figure 1B) using the Prusa i3 MK2 desktop printer with 1.75 mm thick PCL 3D filament. The implant was designed to approximate the tracheal size of a 3.2 kg rabbit with the following dimensions: Height of 20 mm, Inner Diameter of 5.27 x 7 mm and a wall thickness of 0.9 mm.

Figure 1. A) Implant design on AutoCAD software. B) 3D printed PCL implant

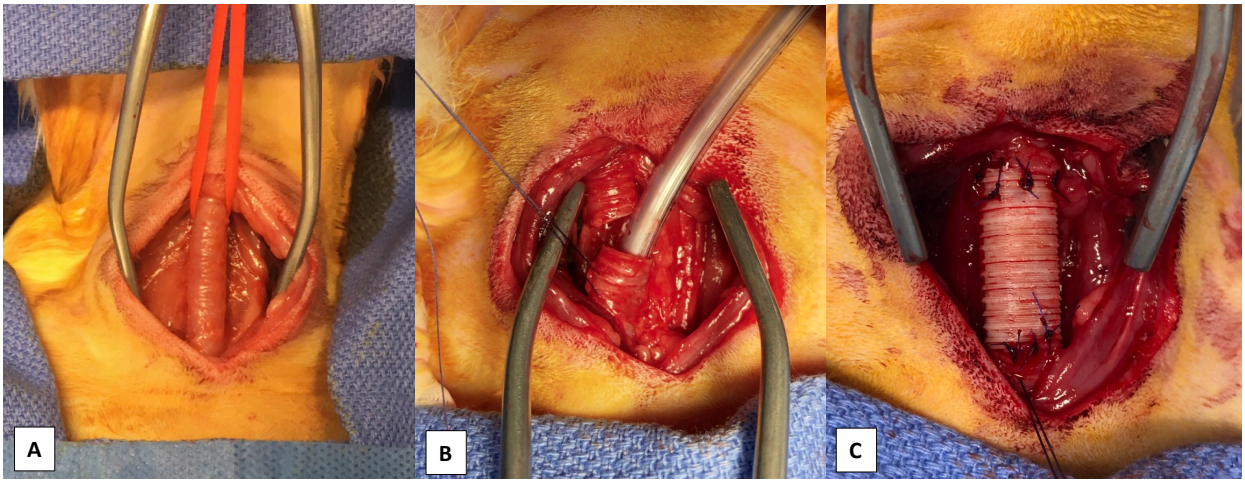


4.3.2. Animals and surgical implantation

Ten New Zealand rabbits were used for our animal model. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the McGill University Health Center. The rabbits were pre-medicated with a combination of ketamine and midazolam and then induced and maintained with isofluorane. They were then intubated, placed in a supine

position and shaved on the anterior surface of their neck and thorax. Rabbits were prepped and draped to maintain sterility. A midline vertical incision was made, and the strap muscles were divided down the midline until the trachea was exposed. Careful blunt dissection was performed circumferentially around the trachea until it was completely freed from the esophagus (Figure 2A). A stay suture was placed in the distal cervical trachea to keep it from retracting into the thorax. The trachea was incised, and a second endotracheal tube was placed into the distal trachea for ventilation (Figure 2B). At this time, a 2 cm segment of the cervical trachea was resected proximally.

Figure 2. Operative Photos. A) The cervical trachea is isolated after division of strap muscles. B) The trachea is incised and an endotracheal tube is placed into the distal trachea. C) PCL implant sutured to proximal and distal cervical trachea.



Anastomosis of the implant to the native trachea was done with 5-0 PDSII (Ethicon) simple interrupted sutures at the proximal end ensuring that the knots were on the external surface of the trachea. We then removed the second endotracheal tube in the distal trachea and the original endotracheal tube was advanced until it was through the PCL implant and into the distal trachea.

The distal segment was then anastomosed with interrupted 5-0 PDSII sutures (Ethicon) around the endotracheal tube (Figure 2C). The strap muscles were approximated with 4-0 Vicryl (Ethicon) and the skin was closed with interrupted 4-0 Prolene stitch (Ethicon).

The rabbits were extubated and brought to the animal care facility where they remained in their isolated cage and monitored daily for respiratory distress, weight changes, and adequate food intake with their regular care. They received enrofloxacin antibiotics for 1 week as well as meloxicam for pain post-operatively for 3 days. Prolene (Ethicon) skin sutures were removed on post-operative day 7.

4.3.2. Evaluation

To progressively analyze the implant over time in the short term. Rabbits were divided into 5 groups, with 2 rabbits in each group, which corresponded to the time points at which the rabbits were evaluated by bronchoscopy (0 [control], 4, 5, 6, 7 weeks post op). Following bronchoscopy, rabbits were euthanized, and their tracheas harvested.

Bronchoscopy

Rabbits were sedated with ketamine, xylazine and acepromazine prior to undergoing rigid bronchoscopy with spontaneous ventilation. Bronchoscopy was performed with a 0º 2.7 mm x 175 mm rigid endoscope and photographs of the airway were taken above the proximal anastomosis. The grade of stenosis for each rabbit was calculated as a percentage of narrowing of the tracheal lumen using the photographs. Using ImageJ software (National Institutes of Health, Bethesda, MD), two reviewers were asked to manually outline the lumen of the stenosed trachea and the lumen of the native trachea separately using the freehand selection tool thereby

computing the relative areas. A similar technique has been described in previous studies[54,55]. This process was repeated 3 times and an average was taken. We determined the percentage of stenosis by calculating the luminal area of the stenosed trachea area over the estimated area of the non-stenosed trachea. Finally, the average of the percentage of stenosis was taken between the 2 reviewers. All reviewers performed well over 20 rigid bronchoscopies in human patients which meets the training requirements to be competent as per the American Thoracic Society, American College of Chest Physicians and the European Respiratory society[56]. It was decided to grade the stenosis based on images as opposed to the more traditional clinical methodology of sizing the airway. This was done as the airway narrowing in the rabbits often quite small and we did not want to risk disrupting the granulation tissue to preserve the histologic features.

Histology

Tracheal segments were fixed in 10% neutral formalin solution and then processed by dehydration through ethanol, cleared in xylene and embedded in paraffin blocks by our histopathology department. Standard 4 μm thick axial segments were taken at 5 points along the specimen, one section on each sides of the anastomosis as well as one section in the center of the PCL implant. Each segment underwent hematoxylin and eosin staining and was analyzed by a pathologist.

4.4. Results

Ten New Zealand male rabbits weighing 2.64-3.35 kg underwent surgical implantation of the PCL implant. Rabbits were euthanized between 0-54 days post-operatively with a median of 30 days (Table 1). Rabbit #6 died of an aspiration pneumonia on post-operative day 5.

Table 1. Rabbit Characteristics

Rabbit #	Weight (kg)	Days of Life	Weeks of life
1	2.87	0	0
2	3.5	0	0
3	2.78	25	3.6
4	2.64	27	3.9
5	2.82	32	4.6
6	3.15	5	0.7
7	3.35	41	5.9
8	3.14	44	6.3
9	3.14	51	7.3
10	3.32	54	7.7

Bronchoscopy was performed on all rabbits except for rabbit #6. All rabbits were found to have significant stenosis at the proximal anastomosis site and whitish fibrinous material was noted on the luminal surface of the PCL implants (Figure 3A-B). The average percentage of stenosis following surgery ranged between 83-98% (Table 2), with a mean of 92.3±6.1%. The Spearman rank demonstrated a strong correlation between the 2 reviewers (Rho = 0.988). There was no apparent trend in the percentage of stenosis over time ($r^2 = 0.0019$).

Figure 3. Examples of photographs taken during rigid bronchoscopy. A) Rabbit #4 – 4 weeks postoperatively B) Rabbit #9 – 7 weeks post operatively

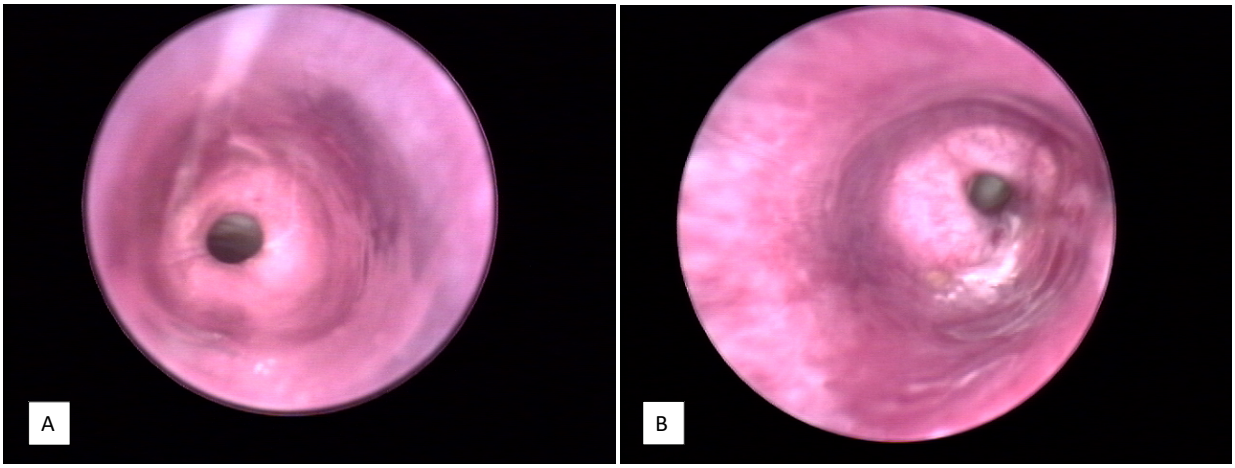
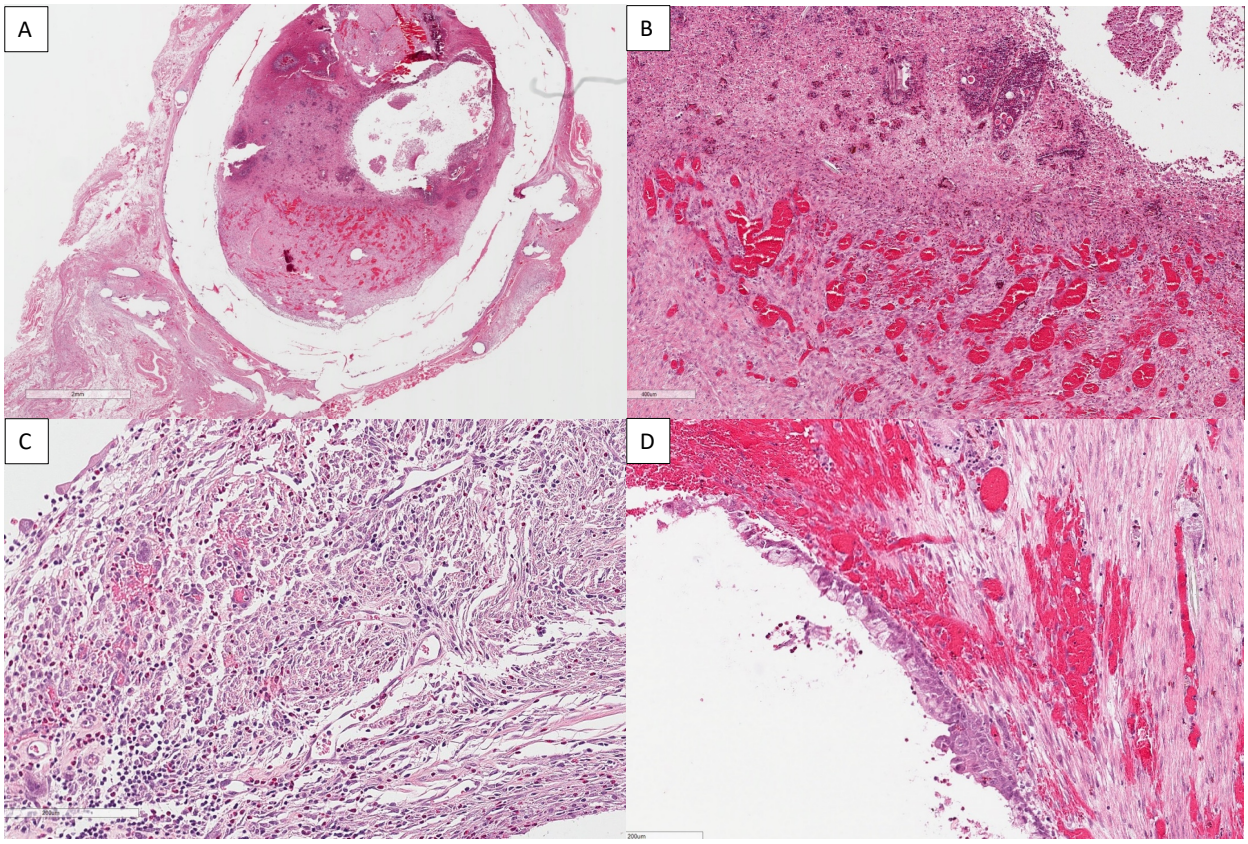


Table 2. Percentage Stenosis Grading

Rabbit #	Reviewer 1 (% stenosis)	Reviewer 2 (% stenosis)	Average (% stenosis)
1	3	3	3
2	1	2	1.5
3	90	92	91
4	97	98	97.5
5	82	84	83
6	-	-	-
7	96	96	96
8	98	98	98
9	83	87	85
10	95	96	95.5

All tracheas were grossly examined once they were harvested. The PCL implants appeared to be well incorporated to the native rabbit tracheas without any evidence of dehiscence or collapse. Histopathological analysis revealed granulation tissue with extensive neovascularization in the tracheal tissue around the PCL implant in all rabbits after 4 weeks (Figure 4 A-B). Mixed inflammatory cells, including neutrophils, lymphocytes, histiocytes, eosinophils, plasma cells were identified (Figure 4C). There was submucosal hyperplasia caused by proliferation of fibroblasts, foci of cartilage were also noted but likely produced by fibroblast like cells. Re-epithelialization was present on the luminal surface of the PCL implant mostly noted near the anastomoses (Figure 4D) but not at the center of the implant. On average epithelium was mixed type (columnar, columnar with goblet cells and stratified squamous epithelium). Based on the qualitative evaluation of the slides, it was difficult to perceive a significant difference in histology between post-operative weeks 4 to 7.

Figure 4. Histologic cross-section of the PCL implant in Rabbit #1 stained with H&E. A) At low power (2 mm), B) Granulation tissue formation (400 μm), C) Inflammatory cells and proliferation of fibroblasts (200 μm), D) Fibrous tissue with partial re-epithelialization (200 μm).



4.5. Discussion

A New Zealand rabbit model was used for this study for several reasons. Some authors argue that it is an ideal model for tracheal surgery because of its similarities to a human infant trachea in terms of structure and size. Rabbits have a long cervical trachea that is easily surgically accessible. In addition, New Zealand Rabbits are easily obtained for research purposes and relatively inexpensive to purchase and house. Rabbits also have a more diverse genetic background compared to other animals which approximates that of humans[57]. PCL implants have recently been used to reconstruct different sized tracheal defects in animal models, with few

studies evaluating complete circumferential reconstructions[50]. To our knowledge, the longest circumferential tracheal reconstruction in a rabbit model with a PCL implant has been 2 cm in longitudinal length which matches the size of our implant[50]. The total length of the rabbit trachea ranges between 5-6 cm, therefore a 2 cm implant corresponds to a reconstruction of 30-40% defect. This length of resected trachea usually corresponds to the limit at which primary anastomosis can no longer be performed in the pediatric population[58].

These results of our study are similar to some studies in the literature despite not having any cellular components to our implants. For example, Kaye et al. impregnated a 2 cm, 270^{o} PCL scaffold with hyaline chondrocytes prior to tracheal reconstruction in 6 rabbits. The subjects were divided into 2 equal groups that were sacrificed at 3 or 6 weeks post-operatively. Their average intraluminal stenosis was 83%[54]. In our study, we calculated an average stenosis of 92%, however our end points were later which may have contributed to further stenosis. We also noted that this can start to occur prior to 4 weeks post PCL implantation. Gao et al. reconstructed tracheal defects in rabbits using a 1.6 cm chondrocyte treated PCL implant in 2 groups, which differed in lengths of time spent suspended in the chondrocyte culture. In 75% of the rabbits, the cause of death was determined to be caused by granulation tissue formation with a mean survival time of 14 and 22 days in each group[58]. Lin et al, implanted a shorter,1 cm long PCL construct seeded with chondrocytes in 6 rabbits. Their rabbits had a mean survival time of 52 days. Similarly, all rabbits experienced narrowing of their airway secondary to granulation tissue with luminal diameters ranging between 1-3 mm.

Airway stenosis secondary to granulation tissue formation appears to be the limiting factor when circumferential tracheal reconstruction is performed using PCL regardless if biologic materials are added to the implant. Granulation formation likely occurs because of the

disruption of ciliated tracheal mucosa that is necessary to clear pathogens and foreign materials. Inhaled microorganisms and other microparticles, if they are not cleared, may induce a continuous inflammatory reaction which will lead to the formation of granulation tissue and subsequent airway stenosis. In addition, the presence of the PCL implant and the sutures used to secure the implant may contribute to a foreign body reaction and therefore further granulation tissue.

Following human airway surgery, patients are often treated with systemic corticosteroids to control edema, inflammation and to reduce granulation tissue formation. They may remain intubated in the intensive care and often a repeat bronchoscopy, with or without intervention on granulation tissue/stenosis, is performed soon after. None of which were performed as part of this study. It was decided to forego the use of corticosteroids to better understand the innate reaction to the PCL implant. Another limitation is the fact that the PCL implants were not customized to the tracheal size of each rabbit, instead a standard size was used. Due to time and funding restrictions we were unable to pre-operatively scan the rabbits and design individualized implants for each rabbit subject. Occasionally during surgery, this meant that the diameter of the PCL implants did not coincide with that of the rabbit's native trachea. This mismatch in diameter may have impeded the migration of epithelium cells which may also have contributed to granulation tissue formation.

PCL is known for its tensile strength however the evaluation of long-term structural integrity of PCL as a tracheal substitute has proven to be difficult as granulation tissue and airway stenosis limit animal survival. Tsukada et al., to our knowledge, were the only study to be able to demonstrate survival of over 1 year in 5 of 11 of their canine subjects after circumferential tracheal defects were reconstructed with copolymer of PCL combined with L-

Lactide however this was supported by a titanium stent and covered with a vascularized omental flap[20].

Synthetic tracheal implants, especially circumferential ones, are also limited by the fact that they would not grow with the subject and therefore the implant itself would be a source of airway stenosis regardless of granulation tissue formation. Theoretically, if this type of implant were to be used in pediatric cases, repeated surgeries could be required to replace it with a larger implant if respiratory symptoms were to arise.

3D printed PCL implants have the potential to be used in reconstructing long segment circumferential tracheal defects. This current study has allowed us to create a baseline evaluation of the PCL implant without any additives. It was demonstrated that it is feasible in the short term, though granulation tissue and stenosis at the anastomoses are the limiting factors that occur prior to 4 weeks of implantation. Further studies would be necessary to tackle this problem. Trials investigating the use of systemic or local anti-inflammatory drugs to reduce the innate reaction against the implant may be beneficial to reduce the amount of granulation tissue formation and allow for longer term evaluation of the implants.

Chapter 5. Overall Discussions

Over the last decade, PCL has been studied for its use in airway surgery in animal models and even in human cases because of its favorable properties. It has been used alone as well as in combination with other polymers and biologic materials to create tracheal splints, stents and tracheal replacements. In this thesis, a scoping review analyzing the literature (Chapter 3) was presented. Based on this review, tracheal splints/stents have had the most success in long term survival in animal models and in human cases. Non-circumferential and circumferential tracheal PCL implants appear to work in the short term; however, they are limited by the development of stenosis and granulation tissue that tends to form at the anastomosis sites. This appears to occur more often in the studies with longer circumferential implants. The review noted that many studies focused on the use of a PCL implant with an additive, either with a polymer or biologic material, without studying, or comparing their design to a bare PCL implant.

Chapter 4 of this thesis describes a study that tried to fill in this gap of knowledge and assess the use of a bare PCL implant to reconstruct a long segment tracheal defect in New Zealand rabbits and to assess the process overtime. Ten rabbits had 2 cm of their cervical trachea resected which was reconstructed with a bare PCL implant of equivalent length. Two rabbits were evaluated by bronchoscopy in each of the following 0 (control), 4, 5, 6, 7 weeks post-operatively. The rabbit tracheas were also harvested for histology. It was found that significant granulation tissue started to form prior to 4 weeks post operatively, as has been shown in previous studies [54,58]. Reconstruction appears to be feasible in the short term; however the stenosis that develops will limit survival using long segmental circumferential implants.

5.1. Unanswered question – Standardizing the Stenosis

Given that this is a new emerging field, there are obviously many unanswered questions that could be addressed in future research. One method of advancing this field of research is to standardize the method to calculate tracheal stenosis in animal models. Stenosis from granulation tissue formation appears to be the major limiting factors in synthetic tracheal replacements. Many different compositions of implants have been evaluated in tracheal reconstruction. Ideally, they should be directly compared in randomized trials; however given the many diverse options, this is not feasible. However, if a standardized method to analyse the degree of stenosis was created, this might permit different studies to be compared. The Cotton-Myer grading system can be used to quantify the degree of subglottic or tracheal stenosis, whereby the largest endotracheal tube, that would generate a leak pressure of 10-25 mmHg, is inserted into the tracheal lumen. Based on the size of endotracheal tube and the age of the patient, a percentage of obstruction could be estimated using their standardized table (Table 1)[59].

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TABLE 1. PERCENTAGE OBSTRUCTION BY ACTUAL ENDOTRACHEAL TUBE SIZE

Patient Age	*Normal ID (mm)*	*Normal OD (mm)*	*Percentage of Obstruction With Actual Endotracheal Tube Size*								
			ID = 2.0	*ID = 2.5*	*ID = 3.0*	*ID = 3.5*	*ID = 4.0*	*ID = 4.5*	*ID = 5.0*	*ID = 5.5*	*ID = 6.0*
Premature	2.0	2.8	0%								
	2.5	3.6	40%	0%							
	3.0	4.3	58%	30%	0%						
0-3 mo	3.5	5.0	68%	48%	26%	0%					
3-9 mo	4.0	5.6	75%	59%	41%	22%	0%				
9 mo to 2 y	4.5	6.2	80%	67%	53%	38%	20%	0%			
2 y	5.0	7.0	84%	74%	62%	50%	35%	19%	0%		
4 y	5.5	7.6	86%	78%	68%	57%	45%	32%	17%	0%	
6 y	6.0	8.2	89%	81%	73%	64%	54%	43%	30%	16%	0%

ID — inside diameter, OD — outside diameter.

This grading scale is the scale that is often referred to in this field. However, it has only been created for human pediatric use.

In the animal studies identified in the scoping review of Chapter 3, many different methods were used for analyzing the degree of obstruction. For example, in the study by Park et al. 2018, the degree of stenosis was classified on a four-point scale, where 0-10% was classified as "no narrowing"; 11-50% as mild narrowing; 51-70% as moderate narrowing; and >71% as severe narrowing[35]. Lin et al. 2009 measured the diameter (mm) of their lumen[33]. Lee et al. 2015 had a binary approach where they stated if there was luminal narrowing or not, on endoscopic and/or radiologic examinations[31]. In the study in Chapter 4, photographs were taken by bronchoscopy to calculate a percentage of narrowing using the ImageJ software (National Institutes of Health, Bethesda, MD). Two blinded reviewers used a freehand tool to outline the lumen of the stenosed trachea and compare it to the lumen of the native trachea thereby computing the relative areas. This process was repeated 3 times for each image and an average was taken. This method was previously described in studies by Kaye et al. [54,55]. All studies described above were performed in New Zealand Rabbits. If a standardized validated method like the Cotton-Myer grading system could be developed for New Zealand Rabbits, studies experimenting with different polymer composition and biologic materials could easily be compared and contrasted. This is one idea that may help to develop an ideal tracheal replacement.

5.2. Other Future directions

Based on the results of this thesis, there are several future directions that may be investigated. Future studies should focus on the prevention of granulation tissue and airway stenosis that tends to occur at the site of anastomosis. Possible use of immunosuppressive agents, or corticosteroids given systemically or applied locally may be a solution. Studies could also evaluate the use of interventions to minimize stenosis development, including stenting or balloon

dilation tracheoplasty. Personalizing the implants to mimic the precise size of the subject's trachea may facilitate the return of the normal mucosal lining, and may limit the formation of granulation. Our group has plans to investigate the testing of balloon tracheoplasty as an option to tackle the stenosis. It is hoped that the results of this thesis will encourage research in this exciting field.

Chapter 6. Overall Conclusions and Claim of Originality

6.1 Overall Conclusions

This thesis set out (1) to review tracheal anatomy, and tracheal pathologies, (2) to review the literature regarding tracheal surgery using PCL in in-invo models, and (3) to evaluate the use of 3D printed PCL implant in the reconstruction of long segmental defects in a rabbit model

Chapter 2 reviewed tracheal anatomy as well as the presentation, etiologies, workup and common treatment options of tracheal stenosis and tracheomalacia.

Chapter 3 presented a scoping review of the literature (manuscript 1) that overviewed the use of PCL in tracheal surgeries in vivo. The review categorized previous studies according to whether they used PCL for (a) full circumferential tracheal reconstruction, (b) partial tracheal wall reconstruction, or (c) as a splint/stent.

Chapter 4 reported the use of a 3D printed PCL implant to reconstruct a 2 cm long circumferential tracheal defect in a New Zealand rabbit model (manuscript 2). It was demonstrated that the PCL implant could be used as a tracheal implant in the short term. However, severe stenosis was generated at the anastomotic sites that occurred prior to 4 weeks post implantation.

6.2 Claim of originality

A unique 3D printed 2 cm long circumferential tracheal implant made of PCL was designed. This is the first study to evaluate this designed PCL implant for the reconstruction of long segmental circumferential tracheal defects, in a New Zealand rabbit model, over time. The study found that the implant was effective in the short term, but the lumen was found to be stenosed at 4 weeks, showing that more research is required to minimize this effect.

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Chapter 8. List of Abbreviations

PCL	Poly(ε-caprolactone)
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
3D	Three Dimensional
H&E	Hematoxylin and eosin
NZR	New Zealand Rabbits
BMSC	Bone marrow stem cells
HTMSC	Human turbinate mesenchymal stromal cells
ECM	Extracellular matrix
MSC	Mesenchymal stem cells