Laryngotracheal

stenosis and reconstruction

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Cover: cross-section of cricotracheal complex of the rabbit with titanium and fascia flap

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Laryngotracheale stenose and reconstructie

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Contents

Chapter 1

General introduction

Laryngotracheal complex

The patency of the larynx and the trachea is sometimes compromised acutely with a direct threat of suffocation or asphyxia. Of old this occurred quite often as a consequence of diphteria and other infectious diseases or as a result of various kinds of exogenous traumata. Infectious diseases for instance, epiglottitis are still among us but are usually handled efficiently by medication and occasionally by intubation.

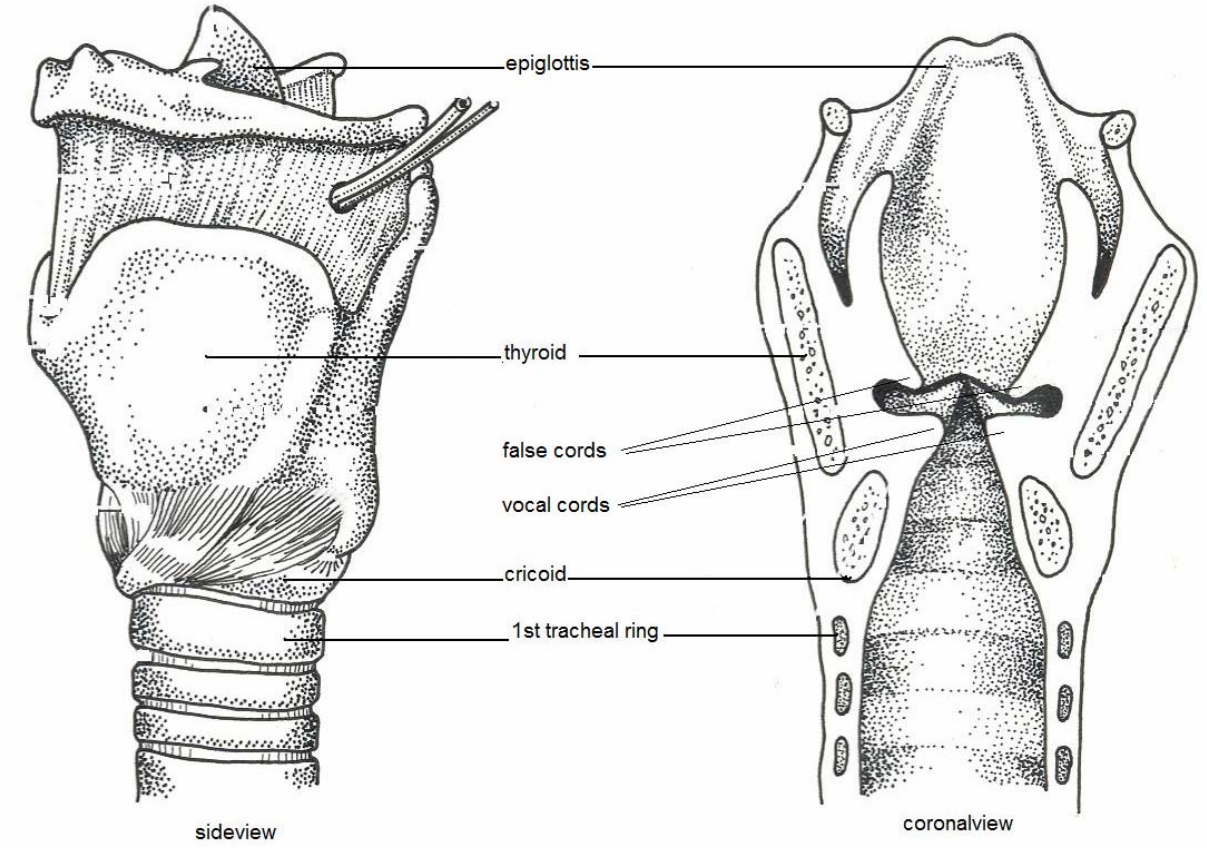

Figure 1.1. Sideview and coronalview of the larygotracheal complex (zakboek Keel-, Neus- en Oorheelkunde)

Just anterior to the esophagus' entrance, the larynx is positioned on top of the trachea. Its main biological function is protection of the airway for which it contains three structures, i.e. the epiglottis with the ary-epiglottic folds, the vestibular plicae (false cords) and the vocal plicae (vocal cords) (Figure 1.1). The only part of the laryngotracheal complex that forms a solid ring is the cricoid cartilage. This inferior part of the larynx sits on top of the tracheal rings which are C-shaped with a membrane at the posterior side. The cricoid stabilizes the larynx and a lot of muscles and ligaments are attached to it to facilitate for speech and swallowing. The cricoid

forms the subglottic area which is prone to trauma during intubation leading to mucosal damage and infection.

This thesis focuses on the anatomy of the cricotracheal complex and on one of its most common pathology known as stenosis. The cricotracheal skeleton consists of hyaline cartilage, covered with a submucosal layer and mucosa that consists of ciliated, pseudostratified columnar epithelium (Figure 1.2A). The cells on top are ciliated, a specialization that allows particles and mucous to be displaced in the direction of the esophagus where particles are finally transported into the stomach. The tracheal epithelium is supported by loose connective tissue, called the lamina propria. It consists of cells with immune function (Figure 1.2B). The cartilaginous structures of the laryngeal skeleton and the trachea are interconnected by fibrous tissue, ligaments and muscles. These cartilaginous structures are important to keep the airway lumen open. The hyaline cartilage matrix has a firm consistency consisting for a large part of water that is retained by a network of collagen (mainly type II collagen) and glycosaminoglycans. The cartilage itself contains no blood vessels but is covered externally by the perichondrium that accomodates the nutritional vascularisation.

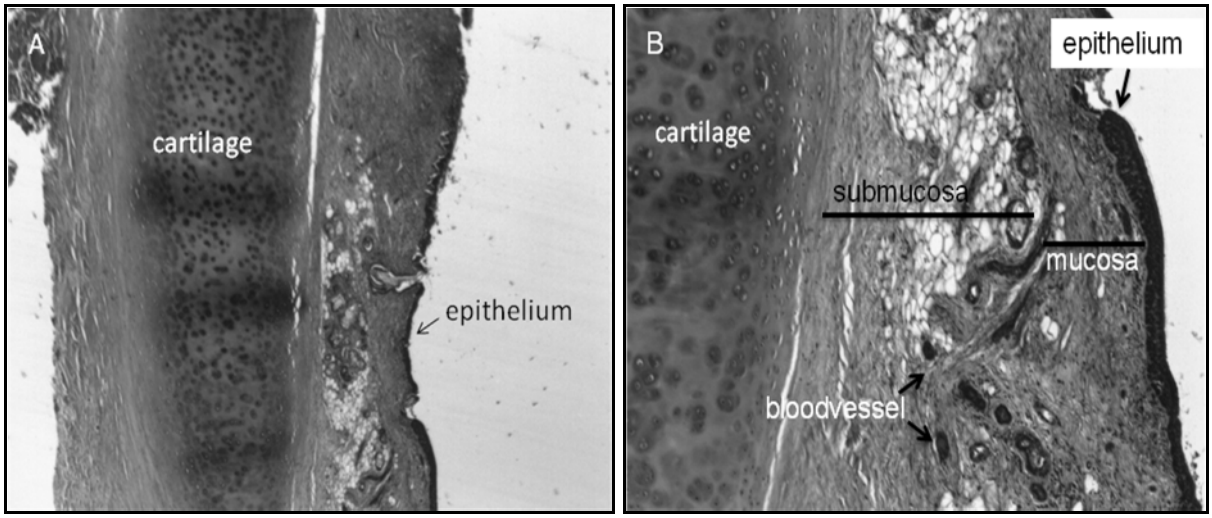

Figure 1.2. Cross-section of human trachea (H & E). **A)** Magnification (original x10) with submucosa and respiratory epithelium. **B)** Magnification (original x40) shows the different layers of the epithelium and cartilage (*see page 113 for color figure*).

In any ENT-department, patients present occasionally with chronic problems associated with decreased patency of the laryngotracheal part of the airway. This anomaly, known as stenosis, sometimes makes an operative procedure necessary

which basically consist of resection of the stenotic part of the laryngotracheal airway and reconstruction of the remaining upper and lower ends to restore its continuity. Stenosis develops if the mucosa gets injured between the tube and the cricoid cartilage ring during intubation or in prolonged intubation. This leads to ulceration and granulation tissue with scar formation and stenosis. For some of these patients, the airway can be reconstructed by an end-to-end anastomosis of the rest of the larynx and the trachea. If the length of the stenotic segment is too long for this option, then a lengthwise incision of the stenotic segment and expansion of the cross-section with autologous cartilage is a viable alternative.

Other categories of patients would include victims of exogenous or endogenous trauma (for instance due to the inhalation of hot gases), benign or malignant tumours and patients with re-stenosis after earlier attempts of reconstruction. The treatment for these patients is resection of the damaged parts of the cricotracheal complex and reconstruction. For some defects, tissue from elsewhere in the body is necessary to rebuild the cricotracheal complex.

In this thesis we evaluated cartilage integration and the survival of cartilage grafts over time after laryngotracheal reconstruction in chronic laryngotracheal stenosis. In order to find new ways to improve laryngotracheal reconstructions in the future we have employed the biomaterial titanium in a series of animal experiments in combination with a vascular carrier and mucosal autografts.

Laryngotracheal stenosis (LTS)

LTS is an infrequent but challenging problem of the airway. Causes of LTS are the sequelae of local tumours, infection and trauma especially those caused by intubation[1]. Nearly 10% of intubated adult patients will subsequently develop LTS[2]. Although the risk increases with prolonged intubation, subglottic injury has been demonstrated within hours after intubation especially the traumatic variant and after tracheotomy. LTS in children is a special problem. Children's stenosis may be congenital (0.3-1%) but are usually acquired (>90%)[3]. Today, more children require intubation and ventilation for extended periods of time following extensive surgery or major trauma. Premature infants are the ones particularly at risk. Between 1 and 9%

of all these intubated pediatric patients will develop a stenosis[4-10]. Prolonged endotracheal intubation or traumatic intubation can cause injury of the mucosal lining leading to ulceration, granulation, cartilage necrosis and scar tissue formation leading eventually to LTS[4-7].

Patients with LTS present with inspiratory stridor or dyspnea. The diagnosis is made by a laryngotracheoscopy. This should be done preferably in a spontaneously breathing patient to determine the vocal cord movement, the exact location, the extent and the nature of the stenosis and to exclude other causes of dyspnea. Its severity (degree of narrowing) is graded on the Myer-Cotton scale[11], based on the percentage of obstruction; grade I from normal to 50% of the surface; grade II from 51% to 70%; grade III from 71% to 99%; grade IV for no detectable lumen. Furthermore if resection is being considered, one should take into consideration the amount of space beneath the glottis for end-to-end anastomosis, the length of the stenosis to be resected and the function of the vocal cords.

Surgical repair of LTS is a challenging aspect of airway management. The principle of subglottic resection in infants and children is basically identical to that of adults although a child's airway is smaller and the postoperative management more difficult. For subglottic and short segment stenosis, repair can be achieved by making use of cartilage interposition grafts in cricotracheal split procedures and with cricotracheal resection (CTR) followed by end-to-end anastomosis[12,13]. The Sophia Children's Hospital has been a major referral center for children with laryngeal stenosis since the 1970's. At present a single stage laryngotracheal reconstruction (SS-LTR) or CTR is usually performed in most cases[14-18]. SS-LTR is an open reconstruction of the airway using cartilage grafts placed in anteriorly and posteriorly split cricoid. The indications for SS-LTR are grade I and II stenosis and grade III stenosis with glottic involvement. In patients with grade IV stenosis or grade III with enough space between the vocal cords and the stenosis, a CTR is indicated[19].

In **chapter 2** we studied the integration of cartilage in laryngotracheal reconstruction in pediatric patients operated on in the last thirteen years using the SS-LTR technique at the Sophia Children's Hospital in Rotterdam, the Netherlands.

In **chapter 3** we analysed the cartilage-cartilage integration after laryngotracheal reconstruction in time. In three patients we performed a MRI of the laryngotracheal complex to learn the fate of the cartilage grafts over time by comparing the postoperative MRI-images 3, 6 and 11.5 years after.

Laryngotracheal defects

Resection of the trachea theoretically can be done safely after resection of up to one-half of the trachea in adults and one-third in infants and small children. The incidence of these cases is not known but most Ear Nose Throat-clinics performing tracheal surgeries see a few such patients annually.

The problem with cartilage when used to reconstruct part of the airway is its integration within the surrounding tissues. Ideally an autologous cartilage graft should integrate with the adjacent cartilage of the trachea or cricoid. However cartilage possesses a poor intrinsic repair capacity. Both in vivo and in vitro studies have shown chondrocyte death in cartilage wound edges[20-23]. This leads to the formation of an acellular band leaving avital tissue in the area where integrative cartilage repair is required. Several enzymatic digestions have been used in an experimental setting in order to increase the initial adhesion of cells or cartilage tissue to host cartilage wound edges[24-26]. Previously we have shown that treatment of an explant with collagenase for 48 hours restored the number of vital chondrocytes in the lesion back to normal values after 14 days of culture[22].

The purpose of the study in **chapter 4** is to shorten the collagenase enzyme treatment period thereby increasing its potential clinical application. We tested the effect of treatment of the graft with (a combination of) hyaluronidase and collagenase in various concentrations and for various periods of time (down to 1 hour). Parameters used were the number of viable cells in the wound edges during culture follow-up in an athymic mice model.

Long segment stenosis involving more than half of the entire tracheal length and re-stenosis after an initial resection and end-to-end anastomosis remains a therapeutic problem since resection of extra tracheal tissue is (usually) not possible. For such

cases, augmentation of the stenotic segment using repair tissues is a valuable option.

Ideally, a tissue for laryngotracheal reconstruction would consist of viable cartilage and respiratory mucosal lining similar to that of the original trachea. Unfortunately there is no such composite tissue elsewhere in the body that meets these requirements. Most of the reconstructive tissues applied clinically lack one or both components leading to a large variation in results. Delaere et al.[27] created a prefabricated and prelaminated repair tissue composed of revascularized elastic ear cartilage with buccal mucosal lining and applied it clinically with satisfactory results. A major drawback of this technique is the long waiting period that is necessary to allow revascularisation of the mucosa and remucosalization of the cartilage graft, before the whole regenerated tissue can be finally transferred to the neck area. Cartilage, by nature is avascular and possesses anti-angiogenic properties, making it a less suitable support tissue for mucosal grafts. This was clearly shown by Delaere et al.[28], when they reconstructed circumferential tracheal defects in rabbits with tubed auricular cartilage pre-wrapped in vascularized fascia. Despite the improvement of vascularisation around the tube, healing through remucosalization only occurred around the anastomotic regions leaving the central portions of the grafts bare and partially necrotic.

The limitations of composite autologous tissues such as cartilage and mucosa stimulated the introduction of various biomaterials. Experiments with solid titanium, Medpor, hydroxyapatite[29-31] or tissue-engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid with or without mucosal lining [32-34] have been performed. The main problem in these studies was the regeneration of the mucosal lining, as reviewed by Tan et al.[35]. In cases of minor defects, reepithelialization from the wound edges occurred, but for larger defects this process of reepithelialization proved to be insufficient. A possible solution to this problem is the use of stable porous biomaterials which allow for the ingrowth of new bloodvessels through their pores which revascularizes the implanted mucosal component and thereby ensure its survival. Any biomaterial used for this purpose should be strong enough to keep the airway lumen open and should not evoke a foreign body reaction or inflammation.

In **chapter 5** we investigated if human and rabbit buccal mucosa will survive on porous titanium in an athymic mice model and in a rabbit laryngotracheal reconstruction model. The specific objective of the first part of the study is to determine if angiogenesis occurs through the porous titanium, allowing graft survival in comparison with cartilage grafts. The specific objective in the second part is to analyze if a construct of porous titanium and buccal mucosa is well tolerated inside the trachea of a living rabbit.

In **chapter 6** full-thickness laryngotracheal defects (cartilage and mucosa) were created and reconstructed with porous titanium in combination with autologous tissues in four different ways in an animal model. The question was if vascular carriers are really necessary when porous titanium is used for reconstruction. Specific questions were; (1) is a fascia flap really needed as a vascular supply, (2) is a two-stage operation needed, the first stage to implant the titanium in order to allow bloodvessels to grow through it and the second stage to implant the titanium with the fascia into the defect, (3) is a mucosal graft really needed to close a defect?

The overall aim of this thesis is to improve laryngotracheal reconstruction by split-procedures and cartilage graft integration. In addition we tested the feasibility of porous titanium with or without a vascular carrier and a mucosal graft as a substitute for cartilage grafts.

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Chapter 2

Results of 60 cases of Single Stage Laryngotracheal Reconstruction in children

Luuk M. Janssen, Hans L.J. Hoeve

Submitted

Abstract

Introduction. Laryngotracheal stenosis (LTS) in children can be treated with single stage laryngotracheal reconstruction (SS-LTR) with cartilage grafting. The objective of this study was to evaluate the surgical outcome of SS-LTR during 13 years, and to find an optimum duration of the direct postoperative intubation period.

Patients and methods. In a retrospective study, the surgical records and postoperative course of 60 children, who underwent SS-LTR in the period January 1994 until December 2006, were analyzed. All patients underwent anterior or anterior and posterior cricoid split and anterior proximal tracheal split with insertion of cartilage grafts performed by the second author. Median age at time of operation, median number of days intubated and median days of hospital stay were recorded. Outcome of surgery was expressed in terms of decannulation rate, stridor, and quality of voice.

Results. A total of 60 children underwent a SS-LTR. Fifty-six patients were diagnosed with an acquired, and four with a congenital stenosis. Forty patients had a grade III stenosis, 17 a grade II, three a grade I. No patient had a grade IV stenosis. The median age at surgery was 2.0 year (0.6-15.8 year). The median time of postoperative intubation was four days with a minimum of one and a maximum of 26. The median stay in the hospital was 13 days (4-103). In 57 patients a sufficient laryngeal lumen was attained. Three patients could not permanently be extubated and still have a tracheostomy. Of the 57 patients without a tracheostomy 51 (89.5%) had no stridor, three (5.3%) had stridor on exertion and three (5.3%) had stridor at rest. The voice was reported by the parents as normal in 35 (61.4%) patients. Twenty-two patients (38.6%) had a hoarse voice. The mean follow-up was almost 2 years with a range of 6 months to 12.6 years.

Conclusion. SS-LTR leads to decanulation in most cases of LTS in children. The majority of patients were successfully extubated after 2-3 days.

Introduction

Laryngotracheal stenosis (LTS) in children is one of the most challenging problems that affect the airway. The stenosis can be congenital (0.3-1% of reported cases) but is usually acquired (>90% of reported cases)[1]. Between 1 and 9% of all intubated pediatric patients who are intubated for more than 24 hours will develop a stenosis[2-8]. Prolonged endotracheal intubation can cause mucosal necrosis leading to ulceration, granulation, scar tissue formation and eventually laryngeal stenosis[4-6,8]. Particularly at risk are premature infants on prolonged ventilatory support and children requiring intubation and ventilation for an extended period following extensive surgery or major trauma.

The Sophia Children's Hospital, a tertiary university referral center in the Netherlands has been treating laryngeal stenosis in children since the 1970's. Several methods have been used in the past. In the earlier days the preferred method was dilatation and intubation. This was later abandoned in favor of the later two stage laryngotracheal reconstruction and stenting. At present laryngeal stenosis are mainly treated with single stage laryngotracheal reconstruction (SS-LTR) or cricotracheal resection (CTR)[9-13]. SS-LTR is an open reconstruction of the airway using cartilage grafts placed anteriorly and posteriorly after a cricoid split. The success rate of this type of surgery is satisfactory but depends on many factors like proper selection of patients, preoperative screenings, patient related factors, type of pathology, timing of surgery and standard of care in the postoperative period.

The aim of this study is review our experience in the management of LTS in children over the last thirteen years and present the surgical outcomes of SS-LTR at the Sophia Children's Hospital in Rotterdam, the Netherlands. Described are the patients, the pre-operative work-up, the surgical and postoperative procedures and the follow-up period. An important aspect was the duration of the postoperative intubation period.

Materials and methods

Patients. The medical records of all children (n=60) who underwent SS-LTR due to an acquired or congenital laryngeal stenosis at the Sophia Children's Hospital in Rotterdam, the Netherlands between January 1st 1994 and 31 December 2006. The chart review included patient data concerning medical history, laryngoscopic findings, tracheotomy, age at surgery, grade of stenosis at the time of surgery, days of postoperative intubation, length of hospital stay, follow-up laryngoscopies and complications.

Pre-operative management. Laryngotracheoscopy in a spontaneously breathing patient was performed to locate the stenosis and to exclude other causes of airway obstruction. The presence of any sign of infection and the degree of inflammatory change were noted. The degree of narrowing was graded on the Myer-Cotton scale[14], on the basis of the percentage of obstruction: grade I to 50% of the surface; grade II from 51% to 70%; grade III from 71% to 99% (any detectable lumen); grade IV total obstruction. Furthermore, the location in reference to the subglottic space (adequate space beneath the glottis for end-to-end anastomosis) and length of the stenosis was noted. In the same session, the function of the vocal cords was assessed. The indications for SS-LTR were grade I and II stenosis and grade III stenosis with glottic involvement. In patients with grade IV stenosis or grade III with enough space between the vocal cords and the stenosis a CTR was performed[15]. All patients were screened for signs of reflux during the endoscopy. Symptoms of reflux are regurgitation, red arytenoids and pachydermia of the inter-arytenoid region. In some patients 24 hour pH metry was performed to exclude a pathological reflux. A pathological reflux was treated with omeprazole or when necessary with fundoplication according to Nissen. All patients were given prophylactic omeprazole to keep the mucosa in the best possible condition as well before as after the operation.

Operative technique. Cartilage grafts were harvested from one of the most caudal costal ribs. Surgical exposure included a horizontal cervical incision with excision of the tracheostomy. The laryngeal skeleton was freed anteriorly and bilaterally. A vertical incision was made through the anterior wall of the cricoid and two to three proximal tracheal rings. The cricoid cartilage was split posteriorly down to the

hypopharynx muscular layer exept for three patients with grade III stenoses. The costal cartilage was molded to fit in the posterior split with the perichondrium facing the luminal side. The patient was re-intubated nasotracheally with a tube that just fits the lumen. This tube is used for 4 days as a stent. Another costal cartilage graft was placed anteriorly with the perichondrium facing the lumen exept for seven patients; they only had a postior graft[11].

Post-operative management. All children were admitted to the pediatric intensive care unit postoperatively. Prophylactic antibiotics (cefuroxim) were given for 7 days followed by co-trimoxazol in a prophylactic dosis for 6 weeks. All patients received anti-reflux medication until 6 weeks after the last extubation. Patients received enteral nutrition through a transpyloric tube. In the first few years the children were paralyzed and heavily sedated with muscle paralyzers. In the later years, the use of muscle paralysis was abandoned and the patients were sedated with midazolam. The patients were preferably not ventilated, and the intubation time could be shortened to 3-4 days.

Laryngotracheoscopy was done 3-4 days after surgery, immediately followed by extubation in most cases. All children received two doses of dexamethasone (0.5 mg/kg, 6 h apart) prior to extubation.

Immediate post extubation stridor was treated with extra oxygen, inhalational steroids and/or epinephrine. When this proved to be insufficient an endoscopy was performed and if necessary a controlled re-intubation by the ENT-specialist.

When there is a sufficient lumen after extubation patients were observed for some days at the nursing department and were discharged when the airway remained stable.

Follow up. Patients were seen at the out-patient clinic 2-4 months after surgery. Stridor was graded as; no stridor at all, stridor during exercise and stridor at rest. The quality of the voice was evaluated by the parents and the physician as normal or with any form of hoarseness or roughness. No objective voice quality tests were obtained. Some patients with voice disorder were seen by our foniatrician and speech pathologist for further guidance.

All patients underwent routinely flexible endoscopy when possible. When there were signs of granulation tissue with stridor or dyspneua endoscopy under general anaesthesia was performed. We did not performed routine endoscopies under general anaesthesia except for the first one after 4 days due to ethical reasons. Only on indication this was performed.

Results

A total of 60 children underwent a SS-LTR. In 56 children (93.3%), stenosis was due to intubation injury. A congenital laryngeal stenosis was present in four children (6.7%). Table 2.1 outlines the general patient characteristics and table 2.2 outlines the co-morbidities of the patients. Median age at reconstruction was 2 years; the youngest child was 7 months and the oldest almost 16 years of age. Forty patients (66.7%) had a grade III stenosis 17 (28.3%) a grade II and three (5.0%) a grade I. There were no patients with grade IV. Forty patients had normal vocal cord movement, six suffered from less movement of one or both vocal cords and in 14 patients the vocal cords were fixated. Fifty-seven patients underwent anterior and posterior split, of these, 50 patients got as well an anterior as a posterior graft and seven patients only got a posterior graft. Three patients had an anterior split with anterior graft.

The median time of postoperative intubation was 4 days with a minimum of 1day and a maximum of 26 days (Figure 2.1). The median post-operative stay in the hospital was 13 days with a minimum of 4 days and a maximum of 103 days. In 57 patients a sufficient laryngeal lumen was attained. This was defined as breathing without stridor at rest.

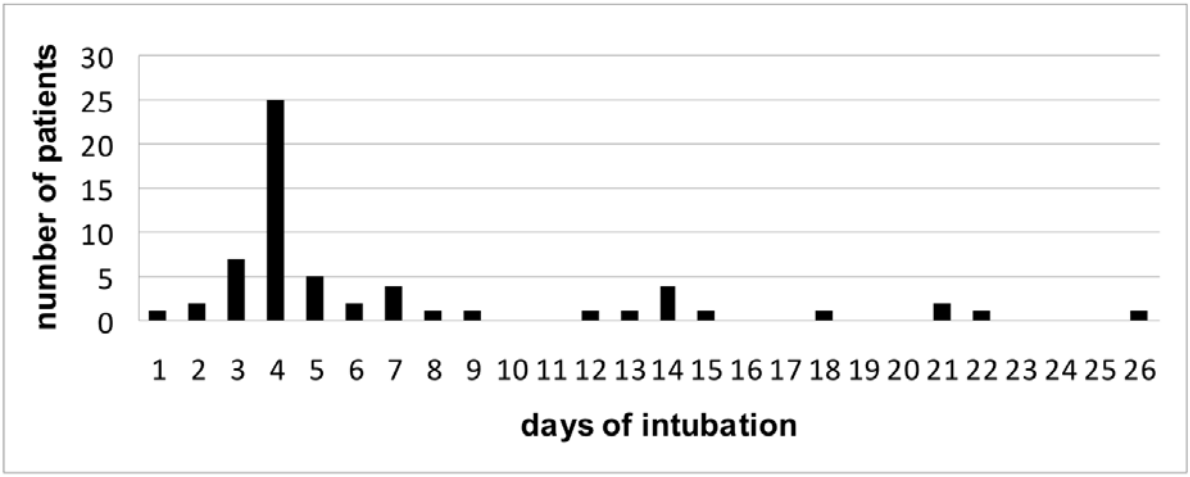

Figure 2.1. Days of intubation (X-axis) and number of patients (Y-axis). Notice that most patients were extubated on day three, four or five (37 patients, 62%).

Median age (months) at time of surgery	23,5 (7-188)
Male / female	26 / 34 (43%/57%)
Intubation injury	56 (93%)
Congenital laryngeal stenosis	4 (7%)
Tracheotomy before operation	47 (78%)
Anterior and posterior split	57 (95%)
Anterior split with anterior graft	3 (5%)
Anterior and posterior graft	50 (83%)
Posterior graft	7 (12%)
Grade I (Myer-Cotton scale)	3 (5%)
Grade II (Myer-Cotton scale)	17 (28%)
Grade III (Myer-Cotton scale)	40 (67%)
Grade IV (Myer-Cotton scale)	0 (0%)
Median days of intubation after operation	4 (range 1-26)
Median days in hospital	13 (range 4-103)
Re-intubation	9 (15%)
Re-tracheotomy	3 (5%)

Table 2.1. Patient characteristics (n=60)

Re-intubation. Nine patients (15%) needed re-intubation after the first extubation. The reasons were granulation tissue (five patients), oedema (two patients) and dyspnoea without any visible disorder (two patients). Three patients (5.0%) eventually needed a tracheotomy because of dyspnoea. These three patients all had a grade III stenosis, one with vocal cord pareses, two with normal vocal cord movement. The other six patients (10%) were finally extubated after various periods of time. Four of these six patients had a grade III stenosis, one patient with vocal cord pareses and two with vocal cord fixation. The other two of these six patients had a grade II stenosis with normal vocal cord mobility.

Prematures	27
Down syndrome	6
Cardiac disease/anomalities	6
Respiratory syncytial virus	4
Trauma	3
Pseudo-croup	4
CHARGE association	1
Pierre Robin sequence	1
Craniolsynostosis	1
Omphalocele/encephalocele	2
Beck-Wiedemann syndrome	1
Esophageal atresia	1
VATER association	1
Multiple dysmorphisms	1
Partial situs inversus	1
Lobectomy	1
Subglottic haemangioma	1
Kawasaki disease	1
Retardation	1
Hyaline membrane disease	1

Table 2.2. Patient co-morbidities

Last out-patient visit. The median follow-up was more than 2 years with a range from 6 months till 12.5 years. Of the 57 decannulated patients 51 (89.5%) had no stridor, three (5.3%) had stridor on exertion and three (5.3%) had stridor at rest. The voice was normal in 35 (61.4%) patients. Twenty-two patients (38.6%) suffered from hoarseness or roughness. Of these patients eight had vocal cord pareses or fixation before the operation.

Discussion

Laryngeal stenosis is one of the most challenging aspects of airway management. There is no single reconstructive technique that can solve all benign stenotic defects of the airway. The choice of the surgical procedure depends on the severity, location and length of the stenosis, as well as the preference and experience of the surgeon.
Although the most severe stenosis are treated with laryngotracheal reconstruction with cartilage graft insertion or CTR and end-to-end anastomosis, there are some minimally invasive techniques available for the mild stenoses (grade I and II), such as laser surgery and balloon laryngoplasty. Laser is used in the management of anterior and posterior glottis webbing with the CO_2-laser being most frequently used[16]. Another endoscopic technique is the balloon laryngoplasty to dilate the subglottic stenosis[17]. These treatments can be combined with the local application of Mitomycin C to prevent post-treatment scar formation[18].
Laryngotracheal reconstructions are indicated in less severe stenosis and posterior transglottic stenosis. The goal is to create a larger lumen which can be achieved by expanding the stenosis after anterior and posterior split. In the past, these techniques involve placement of intraluminal stents. In SS-LTR the only ‘’stenting’’ is the temporary use of an endotracheal tube[11,19]. The cartilage grafts keep the expanded cricoid and trachea open. CTR is indicated for cases of severe subgottic stenosis (grade III and IV). In this operation the stenotic segment is removed and the trachea is anastomosed to the subglottic space. In stenosis without residual subglottic space we think that a SS-LTR is the better option. Many different opinions exist about which technique should be used for which case as reviewed by Bailey et al. in 2002[15].

Of all 60 patients in our study group 57 (95%) had a sufficient laryngeal lumen at discharge. This high success rate is due to several factors. In our department we strictly adhere to a standardized work-up and follow-up for all of our patients.

A laryngotracheoscopy is only performed by an experienced otolaryngologist. The degree of maturation of the scar tissue is always evaluated and the operation is performed only when the stenosis is mature and there were no signs of inflammation (redness, oedema and granulation). Strict adherence to this rule had contributed to our good results.

Other causes of upper airway obstruction which sometimes could have possibly been the initial reason for intubation, such as a narrow nose, narrow choanal region, retrognathia, macroglossia, vocal cord dysfunction, supratracheostomal collapse, or tracheomalacia when noted, should always be dealt with prior to the SS-LTR[20].

The median intubation duration after laryngotracheoplasty in this study was 4 days although shorter intubations appeared to be sufficient. One single patient who extubated himself accidentally on the first postoperative day, was not re-intubated and recovered well. The same holds for the patients extubated after 2(two patients) and 3(seven patients) days. This suggests that it is possible to limit intubation to 3 or even 2 days.

As a matter of course a high-level intensive care unit allowing for necessary optimal care immediately after the operation up to the phase of early extubation is critical for success of the treatment. To avoid unnecessary re-intubation with the chance of damaging the reconstruction, a multidisciplinary approach and a proper communication between the otorhinolaryngologist and intensive care specialist and nursing staff are a must.

In our series there were no fatalities. Nine patients needed re-intubation because of granulation tissue(five patients), oedema(two patients) or dyspnoea(two patients) without manifest anatomical disorders. Three patients (5.0%) eventually needed a new tracheotomy because the laryngeal obstruction, did not respond to conservative measures or re-intubation.

At the last follow up visit 51(85%) of the patients had no stridor at all. One-third of all patients had voice disorders. As the primary goal of laryngotracheal surgery is to create a sufficiently patent airway it is only in the course of the last few years that more attention has been paid to voice outcome. Baker and Kelcher[21,22] stress that

more attention should be paid to voice impairment scales after laryngotracheal reconstructions.

Comparing the results of various treatments is difficult, due to ill defined criteria of success. In many studies decannulation has been the main parameter of success. Others define success based on functional outcome, i.e. dyspnoea and phonation. Cotton reported an 86% success rate for SS-LTR in his study in 1992[23] and a decannulation rate of 90% for all techniques used in another review[24]. Lusk had one failure out of 19 patients in his first series of SS-LTR in 1991[19]. Seid had 12 successful extubations after SS-LTR out of 13 patients[11]. Triglia in Marseille reported 95% decannulation rate in 98 children who were operated with different techniques including 58 laryngotracheal reconstructions[25]. The decannulation rate of 95% in our study is comparable with the decannulation rates found in literature after SS-LTR or CTR[26-29].

Conclusion

Our results compare well with those of other centers in terms of decannulation rate. SS-LTR led to decannulation in most children with laryngeal stenosis. SS-LTR appears to be an effective and safe operation in selected cases of LTS in children. Furthermore, our series suggest that intubation time after the operation can be as short as 2 or 3 days.

Acknowledgments

We thank Prof. Dr. Louw. Feenstra, Dr José .A. Hardillo and Dr Gerjo .J.V.M. van Osch for their valuable comments on earlier versions of the manuscript.

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Chapter 3

Magnetic resonance imaging of cartilage grafts used in laryngotracheal reconstruction

Luuk M. Janssen, Maarten H. Lequin, Piotr Wielopolski, Hans L.J. Hoeve

Submitted

Abstract

MRI of cartilage grafts used in three children who were treated with single stage laryngotracheal reconstruction (SS-LTR) for laryngotracheal stenosis was performed respectively 3, 6 and 11.5 years after the initial operation. The indication for MRI was airway obstruction with suspicion of displacement of the cartilage grafts. In all three patients the grafts were visible on MRI. There was no cartilage-cartilage connection or fusion, but the cartilage grafts were connected to the surrounding structures with what we believe is fibrous tissue. No signs of resorption were seen.

Introduction

At the Sophia Children's Hospital, a tertiary university referral center, the majority of patients with laryngotracheal stenosis (LTS) are treated with single stage laryngotracheal reconstruction (SS-LTR), an one-stage open reconstruction of the airway with anterior and posterior cricoid split and insertion of autologous costal cartilage grafts to expand the lumen.

Cartilage is an avascular structure and when harvesting a piece of cartilage chondrocytes will die in the wound edges. This leads to the formation of an acellular band with avital tissue with no potention to heal adequately[1]. The cartilage will be connected with fibrous tissue to surrounding structures. Zalzal studied autologous cartilage graft survival in rabbits in 1986[2]. He found 100% graft survival with autologous elastic cartilage. An important factor in survival of the cartilage could be the perichondrium. In our operation technique the cartilage is used with the perichondrium facing the lumen of the larynx and trachea. The posterior graft is positioned between the cricoid halves and covered with fibrin glue. The anterior cartilage graft is stitched to the incision edges. Imaging of cartilage with MR is not extensively described yet in the pediatric literature. MR imaging with high resolution gadolinium enhanced T1-weighted spin echo sequence of tracheal cartilage grafts in rabbits was shown by Delaere et al. in 1999[3]. In our study we used an MRI technique with special surface coils to give a high resolution of the cricotracheal complex to make the cartilage visible.

The aim of our case-study is to examine the fate of the cartilage grafts after various periods of time: are signs of resorption present? And how does the graft integrate with the surrounding tissue?

Patients

Patient I. A boy with a congenital stenosis grade III according to Myer-Cotton[4] and Kawasaki's disease underwent a tracheotomy at the age of 3 months. One year later a SS-LTR was performed with an anterior and posterior split and grafting. The postoperative period was complicated by severe airway obstruction caused by

granulation tissue, displacement of the anterior graft, and collapse of the anterior tracheal wall. A tracheostomy was necessary 3 weeks after the operation. MRI was performed 3 years after the operation (Figure 3.1)

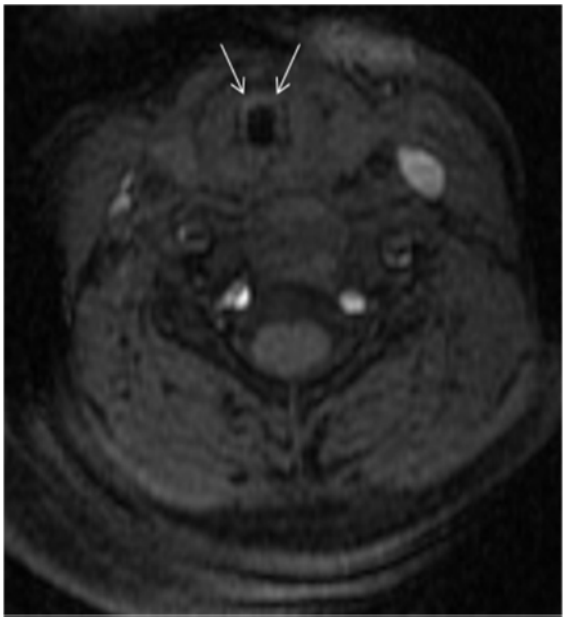

Figure 3.1. This axial T1 weighted image shows the larynx. The anterior graft is more intense compared to the cricoid (white arrows). At this more proximal level the anterior graft was in place.

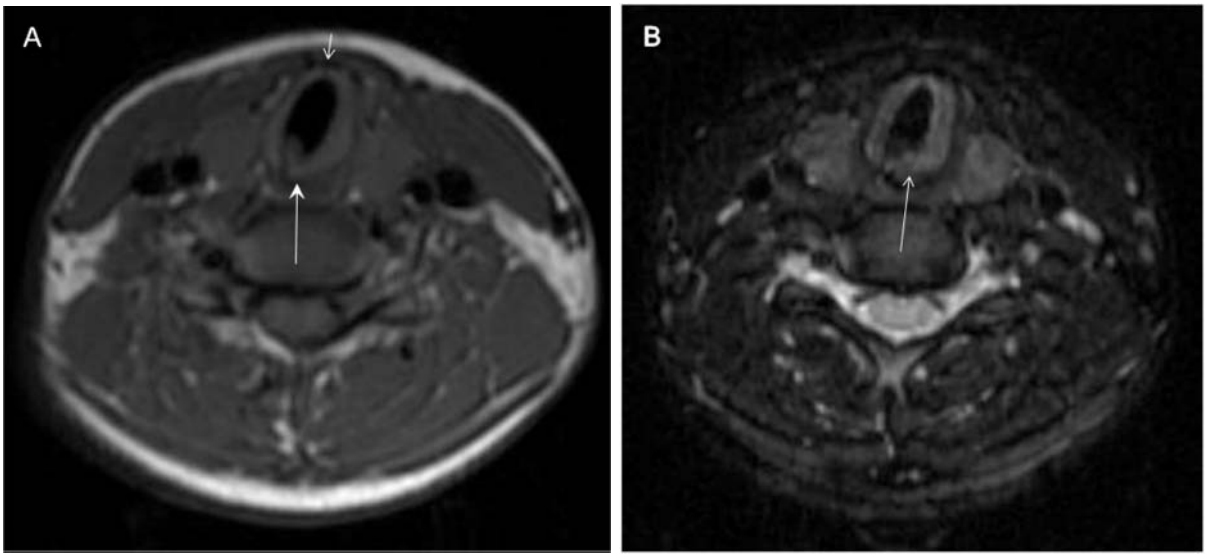

Figure 3.2. These axial T1 weighted (**A)** and T2 weighted (**B**) image shows a larynx 6 years after the SS-LTR with anterior and posterior cartilage grafts. The small arrow is pointing at the anterior split, just above the level of the anterior graft which is not visible in this image. The bigger arrow is pointing at the posterior graft which is displaced a little into the laryngeal lumen.

Patient II. A 13.5 years old girl underwent SS-LTR for a posterior glottic and subglottic grade II stenosis following prolonged intubation and ventilation after her premature birth. She had no tracheostomy, but moderate symptoms of airway obstruction. During the years after the operation stridor re-occurred. A MRI was performed 6 years after the SS-LTR (Figure 3.2).

Patient III. A girl with Down syndrome and a subglottic stenosis grade III caused by prolonged intubation and ventilation underwent SS-LTR at the age of 22 months. The cricoid was split anteriorly and posteriorly with a mucosal graft posteriorly and a cartilage graft anteriorly. Over the years several endoscopies were performed because of signs of airway obstruction caused by a mild rest stenosis. A MRI was performed 11.5 years after the operation to see how the cartilage graft was situated in the cricotracheal complex (Figure 3.3).

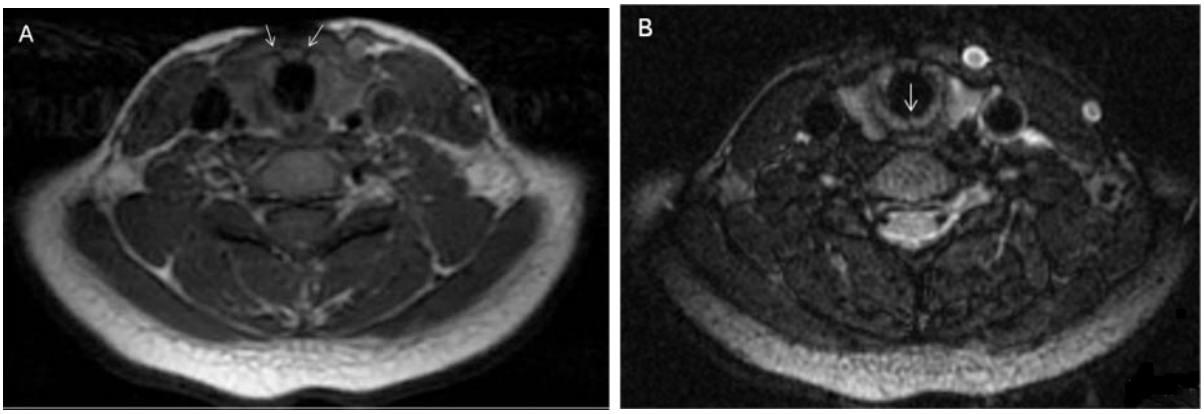

Figure 3.3. These axial T1 weighted (**A**) and T2 weighted (**B**) image shows the larynx 11.5 years after SS-LTR with an anterior and posterior cricoid split (white arrow in B) and a cartilage graft anteriorly. The anterior graft shows no resorption and is well depicted (white arrows in A).

Magnetic Resonance Imaging of the laryngotracheal complex. We performed a MRI of the laryngotracheal complex, using a MR larynx protocol on a 1.5-T GE EchoSpeed scanner (GE Medical Systems, Milwaukee, Wis.). This protocol included axial, sagittal and coronal T1-weighted (spin echo, TR/TE 460/ 17 ms) and T2-weighted sequence (fast spin echo, TR/TE 4460/ 81 ms) with a slice thickness of 4 mm and 0.4 mm gap in the axial orientation and 3 mm and 0.3 mm gap in the other directions. In patient II we used a quadriture coil, normally used for knee imaging. In patient I and III, we performed a MRI with the same MR larynx protocol and an additional 3D T1 weighted gradient echo sequence with and without fat saturation,

using a special surface coil, designed for imaging the carotid arteries. The slice thickness was 1.6 mm in axial planned scans and 1 mm in coronal planned scans.

Discussion

This is the first case study, to visualize cartilage grafts using a MRI in children after SS-LTR. In all three patients the grafts were visible with the MRI technique we used. No resorption was seen. There was no cartilage-cartilage connection but the cartilage grafts were in place and connected to its surrounding with what we believe is fibrous tissue. This confirms earlier studies in animals and in humans[2,5,6]. A possible explanation could be that perichondrium prevents infection of the cartilage and thereby resorption [2]. The survival of cartilage covered with perichondrium is believed to be much better because perichondrium prevents infection of the cartilage[2]. Another important factor in graft survival is given by Albert et al. in 1989[7]. They describe that cartilage damage during the operation promotes granulation with ingrowth of granulation tissue in the cartilage which leads to resorption.

We have shown that cartilage grafts can be made visible with a special MRI-technique presented here and we have shown that cartilage grafts with perichondrium will not resorp in time.

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Chapter 4

Short-duration enzymatic treatment promotes integration of a cartilage graft in a defect

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Abstract

Objective. Surgical manipulation of cartilage tissue is associated with chondrocyte death in the wound edges that hinders integration. The objective of this study was to evaluate the effect of a short course of treatment of a cartilage graft with a combination of hyaluronidase and collagenase on chondrocyte density and integrative capacity.

Methods. Cartilage explants were treated with enzymes for various time periods and at various concentrations. A central core was punched out of a larger explant, treated with enzymes, re-implanted and placed subcutaneously in athymic mice. The number of chondrocytes in wound edges was counted and the integrative capacity of the grafts was evaluated histological.

Results. Treatment with collagenase for 48 hours led to a significant increase in the number of vital chondrocytes and restored it to normal after 14 days of culture. Treatment with hyaluronidase and collagenase for 48 hours further increased chondrocyte densities to supra-normal values. Shortening the treatment to 1 hour restored the chondrocyte density to normal after 14 days of culture. In vivo integration experiments showed increased chondrocyte densities in treated wound edges and extra-cellular matrix fibers crossing over from enzyme-treated parts to untreated parts.

Conclusion. Short-duration treatment of a cartilage graft with a combination of hyaluronidase and collagenase increases cell density at wound edges and promotes integrative repair.

Introduction

Cartilage surgery constitutes a major part of several surgical disciplines such as orthopedics, otorhinolaryngology, and plastic and reconstructive surgery. In the field of otorhinolaryngology and head and neck surgery, cartilage is used primarily in laryngotracheal reconstructions and operations involving the auricle and the nose. The success of the use of cartilage grafts in head and neck surgery depends on the quality of integration of the graft with the surrounding cartilage structures[1-3]. Depending on the location in which the graft is used, failure of repair can result in severe airway obstruction or gross deformation of the ear or nose. As such, attempts should be made to ensure a significant and durable repair. However, cartilage is known to possess a poor intrinsic repair capacity. Both in vivo and in vitro studies have shown chondrocyte death in cartilage wound edges[2-5]. This leads to the formation of an acellular band leaving avital tissue in the area in which integrative cartilage repair is highly required. The cells adjacent to this area would normally become increasingly active and undergo proliferation; however, they are not able to repair the wound because of the interposition of this acellular tissue and the inability of chondrocytes to migrate through the tight extracellular matrix[2,3,6]. The lack of matrix-producing cells in the area in which integration is required may be the limiting factor.

Several enzymatic digestions have been used in an experimental setting in order to increase the initial adhesion of cells or cartilage tissue to host cartilage wound edges[7-9]. Previously, we showed that treatment of an explant with collagenase for 48 hours restored the number of vital chondrocytes in the lesion edges back to normal values after 14 days of culture[4]. These results indicate positive effects of this treatment on the process of integrative cartilage repair.

The purpose of the present study was to shorten the collagenase enzyme treatment period, thereby increasing the potential clinical application of the treatment to surgery. Shortening the treatment period, however, would eventually require potentiating the effect of collagenase with another enzyme. Removal of proteoglycans with the enzyme hyaluronidase could facilitate the action of collagenase[4]. We tested the effect of treatment with a combination of hyaluronidase and collagenase in various concentrations and for various periods of time (down to 1

hour) on the number of viable cells in wound edges during culture follow-up. Finally, the effect of enzyme treatment of the graft only on integration with surrounding cartilage was evaluated by subcutaneous implantation of constructs in athymic mice.

Methods

Testing enzyme treatment protocols. Full-thickness articular cartilage samples were harvested from the metacarpophalangeal joints of 6 months old calves, within 6 hours after slaughter, under sterile conditions with 4 mm dermal biopsy punches (Stiefel, imported by Bipharma, Weesp, the Netherlands). Explants were collected in culture medium and randomly divided into treatment groups. Before to culture, cartilage was either kept at 37 °C in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Gibco, Grand Island, New York, USA) supplemented with 2% fetal calf serum (FCS) or treated with highly purified collagenase VII (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and/or hyaluronidase type I-S from bovine testes (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) in DMEM/Ham's F12 supplemented with 2% FCS. Various enzyme concentrations were used (hyaluronidase 0.1% and 3% and collagenase 10 U/mL, 300 U/mL, 600 U/mL) with treatment time periods of 48 hours and 1 hour (Figure 4.1). The extracellular matrix of cartilage mainly consists of proteoglycans and collagens. Hyaluronidase was used to remove proteoglycans and collagenase was used to remove collagens. When hyaluronidase is used first, the cartilage becomes more susceptible to collagenase treatment. The explants were subsequently washed thoroughly with medium and cultured for 14 days at 37º C in a humidified incubator 5% carbondioxide in separate wells with DMEM/Ham's F12 medium supplemented with 10% FCS and 25 µg/mL ascorbic acid 2-phosfate (Sigma-Aldrich Chemie BV), 0.5 µg/mL Fungizone, and 50 µg/mL gentamicin. The medium was changed three times a week. Finally, the explants were evaluated histological.

Integration experiment. Full-thickness explants were harvested using 8 mm dermal biopsy punches. A central core was punched out using a 3 mm punch to divide the 8 mm diameter discs into final explant rings (8 mm outer diameter) and explant cores (3 mm outer diameter). The inner cores were either treated for 1 hour with a

combination of 3% hyaluronidase and 300 U/mL collagenase suspended in medium (DMEM/Ham's F12) supplemented with 2% FCS or left in medium with 2% FCS. Then the samples were thoroughly washed in culture medium and the 3 mm inner-cores were re-implanted in their accompanying 8 mm outer ring. These constructs were then implanted in separate subcutaneous pockets on the back of athymic mice (BALB-C nu/nu; Harlan, Horst, the Netherlands) with approval of the local animal ethical committee (Protocol No. 126-01-01). Each mouse carried four constructs: two in the back and two in the front (n=12 for the enzyme-treated group and n=6 for the control group). After 5 weeks the mice were sacrificed and the constructs were harvested (Figure 4.2).

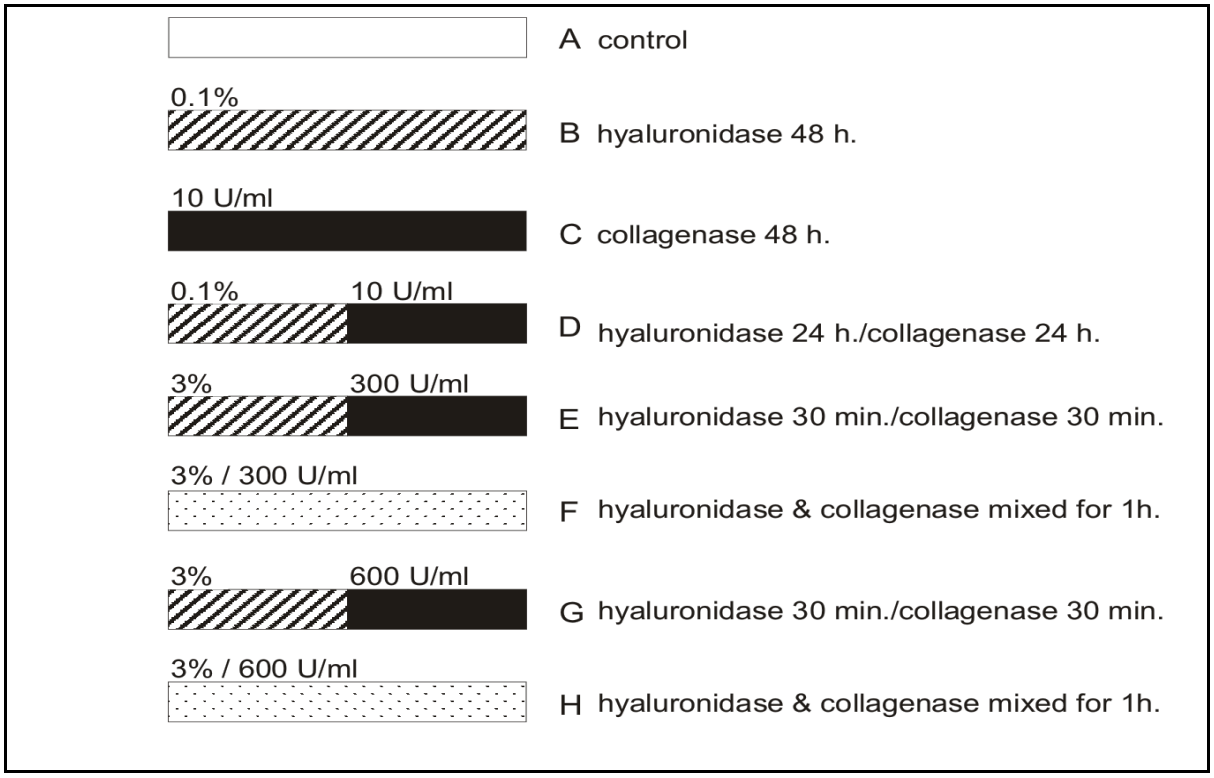

Figure 4.1. Schematic overview of the 7 different enzymatic treatment protocols used. Before culture, full-thickness articular cartilage explants were kept in culture medium or were treated with highly purified collagenase and/or hyaluronidase in medium with 2% fetal calf serum. Various enzyme concentrations, marked A-H, were used in treatment time periods of 48 hours and 1 hour.

Histology. To evaluate the effect of enzyme treatment we focused on evaluating the number of vital cells in the wound area. Previous studies have indicated that the number of vital cells in the wound area is an important prerequisite for integrative cartilage repair[4,6]. Therefore, cartilage discs from all experiments were fixed in 4% phosphate-buffered formalin, processed, and embedded in paraffin for histology. By

means of a microtome, 6 µm paraffin sections with at least 70 µm interval were cut, mounted on slides and stained with heamotoxylin and eosin for evaluation of chondrocyte viability and quantification of cell density. Nuclear and cytoplasmatic changes as described by Kim and Song in 1999[10] were used to judge cell viability or cell death. Only cells with visible nuclei being evaluated. The wound edge was defined as a band of 150 µm from the lesion edges. The central part of the explant, one mm distance from the lesion's edge was defined as unwounded cartilage. Vital looking chondrocytes were counted in the superficial zone (100 µm from the surface down), the deep zone (150 µm from the base up), and the middle zone (between the superficial and deep zones)[11]. Cell counts were calculated per square millimeter of both unwounded and wounded areas at 400 x magnifications with a grid containing 50 x 50 µm boxes. We used the average of two to four different sections counting the chondrocytes in two of each zone.

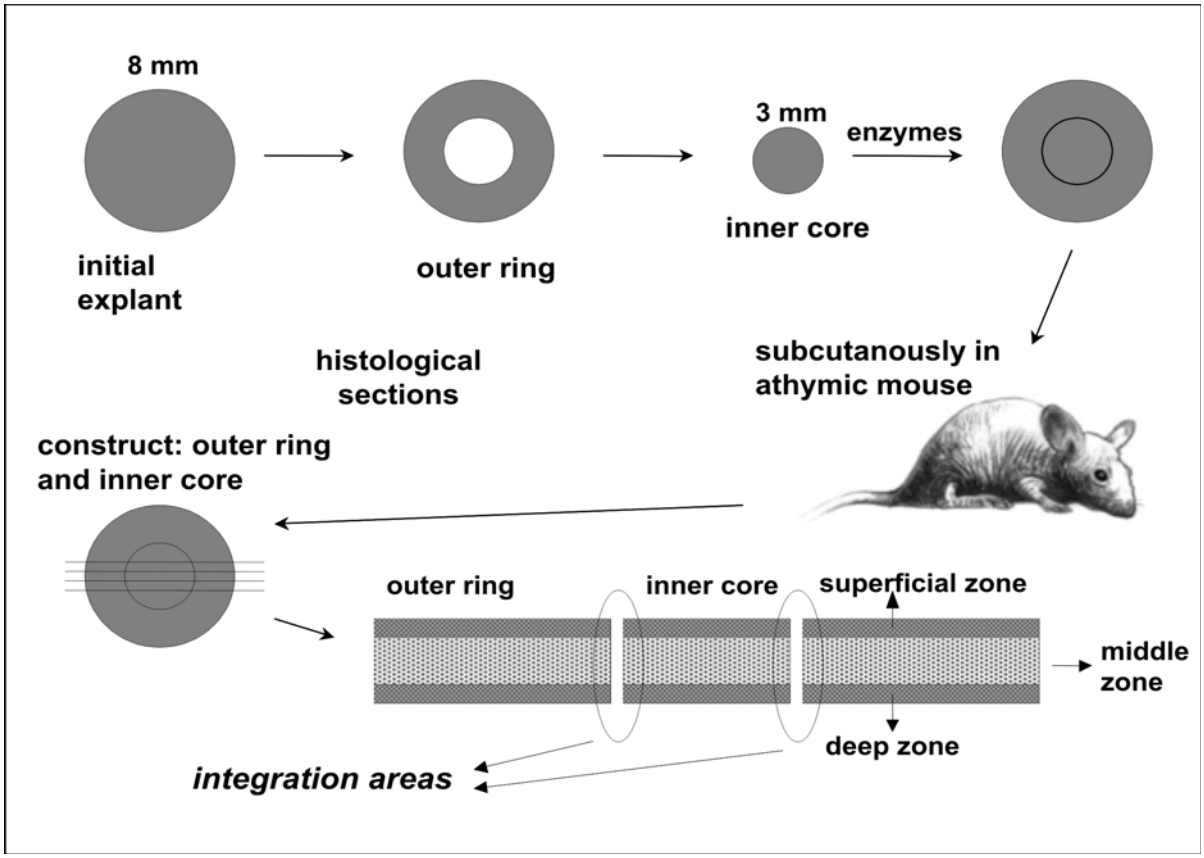

Figure 4.2. Schematic representation of the integration experiment. Full-thickness 8 mm cartilage explants were harvested and 3 mm central core was punched out, resulting in outer ring and inner core explant. Inner cores were treated with enzymes or kept in control medium. The inner cores were subsequently re-implanted into outer rings and implanted in athymic mice for 5 weeks. Histologic sections were cut as indicated. Counting of viable cells was done in wounded and unwounded areas, in superficial, middle and deep layers.

In the integration experiment, viable cell counts of the enzyme-treated area (wound edge of the inner core) were compared with the non-enzyme treated area (outer wound edge of the outer ring). In addition, the percentage of the inner core interface area that was connected to the outer ring was scored in two to four different sections and an average was calculated for each construct. The presence of integration of 5% or more of the interface area was calculated resulting in an incidence of integration. Integration less than 5% was considered as no integration.

To evaluate the matrix in the integration area, we stained sections with 0.04% thionine in 0.01mol/L aqueous sodium acetate or with 0.1% Sirius Red F3BA in a saturated picric acid solution. Furthermore, cryosections were stained for procollagen type I (M38 1:100; Developmental Studies Hybridoma Bank [DSHB], maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore Maryland, and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under contract N01-HD-6-2915 from the national institute of child health and human development) and collagen type II (II-II6B3 1:100, DSHB). Pretreatment with 1% hyaluronidase (Sigma-Aldrich Chemie BV) was used to unmask the epitopes. All primary antibodies were previously complexed with goat Fab-fragment against mouse conjugated with alkaline phosphatase (GAMAP, 1:400, Immunotech, Marseilles, France) at 4°C overnight. After coupling 0.1% normal mouse serum was added 2 hours before application to the sections to capture the unbound GAMAP, after which the antibody solution was used on the slides for 2 hours at room temperature. The sections were subsequently incubated for 30 minutes with alkaline phosphatase anti-alkaline phosphatase (APAAP, 1:100, Dakopatts, Copenhagen, Denmark). New Fuchsine substrate (Chroma, Kongen, Germany) was used for color development. Negative controls were subjected to the same protocol with omission of the primary antibody.

Statistical analysis. We finally had seven to 18 samples for each experimental group of the in vitro experiment, 12 samples for the enzyme-treated group and six for the control group of the in vivo experiment. The results are expressed as mean ± SD. Differences between enzymatic treatment groups, unwounded areas and control groups were calculated with the Kruskall-Wallis test. Differences between individual

groups were calculated using the Mann-Whitney U test. For the integration experiment, the incidence of integration was calculated using the Fischer's exact test.

For all tests, p values of less than or equal to .05 were considered statistically significant.

Results

After harvesting the explants, we observed cell death at the wound edges (Figure 4.3) of the control explants, leading to a decrease in the number of vital chondrocytes as compared to unwounded cartilage after 14 days of culture (Figure 4.4, group A). This decrease was significant in superficial and middle zones. Treatment for 48 hours with hyaluronidase had no positive effect (Figure 4.4, group B). After treatment for 48 hours with highly purified collagenase type VII (Figure 4.4, group C), a significant increase in the number of vital chondrocytes at the wound edge was seen after 14 days in culture in comparison to the untreated control group in the wounded part (group A). This resulted in an amount of vital chondrocytes similar to that in the unwounded cartilage at time point zero. Treatment for 24 hours with 0.1% hyaluronidase followed by 24 hours with 10 U/mL collagenase (Figure 4.4, group D) led to an even larger increase in vital chondrocytes. After 14 days of culture, the number of vital chondrocytes in the treated wound edges was significantly higher than in the unwounded cartilage at time point zero. Reducing the treatment time to 1 hour (Figure 4.4, groups E-H) in combination with higher concentrations of hyaluronidase and collagenase also led to a significant increase in vital chondrocytes in comparison to untreated wound edges (group A) in all treatment protocols tested, particularly in the superficial and middle zones. No significant differences were found between treatment with 300 U/mL collagenase (group E) and 600 U/mL collagenase (group F). Likewise, no significant differences were found between adding hyaluronidase and collagenase subsequently for 30 minutes each (group G) and adding them combined for 60 minutes (group H). Furthermore after 14 days of culture, the chondrocyte densities in treated wound edges were not significantly different from unwounded cartilage at time point zero.

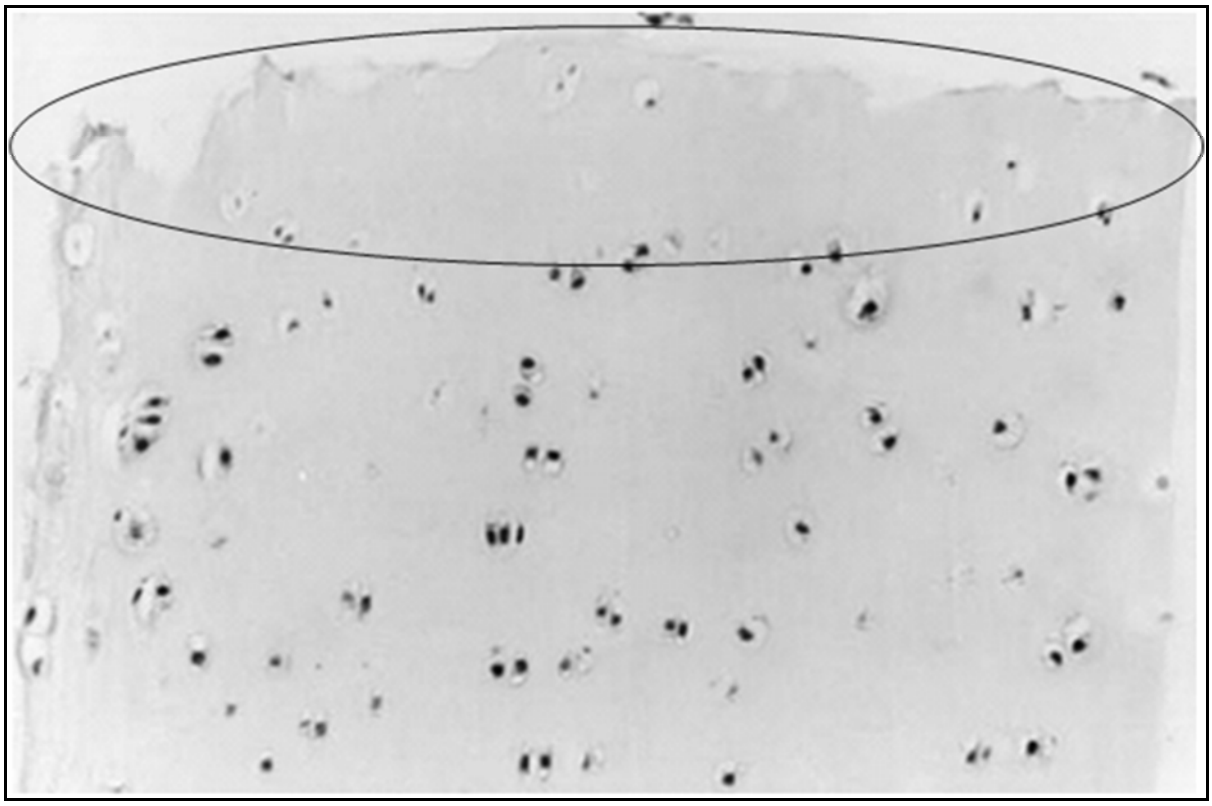

Figure 4.3. Chondrocyte death in the wound edges (H&E, original x100). Fourteen days after wounding acellular band is visible at wound edge (circled). Left is the superficial layer and right is deep layer (*see page 113 for color figure*).

In the in vivo integration experiment, the number of vital chondrocytes in the wounded enzyme-treated area was higher than in the untreated area (Figure 4.5 and 4.6). In the treated group, the inner cores were significantly more often integrated with the outer rings than the untreated control group (80% versus to 46%). The percentage of the interface area that was integrated was 53% ± 19% for the treated group and 39% ± 23% for the control group. On histological cross-section of each sample, two integration sides appeared. When we split the treated group into a ‘’better integration side’’ and a ‘’worse integration side,’’ the averages respectively, were 75% ± 19% and 26% ± 31%. The matrix in the integration area stained positive with Sirius red and moderately positive with thionine. The connection between the enzyme-treated inner core and its untreated outer ring is formed by fibers (that stain with hematoxilin and eosin, thionine and Sirius red) crossing over from the treated to the untreated part (Figure 4.6C). Immunohistochemical staining indicated that cells and the tissue at the interface are positive for collagen type II. When the integration is either good or totally absent, collagen type I can be found in only a few cells at the

interface. However, in a few cases in which a gap occurred that was filled with ingrowing tissue from the surroundings, this interface tissue was positive for both collagen I and II.

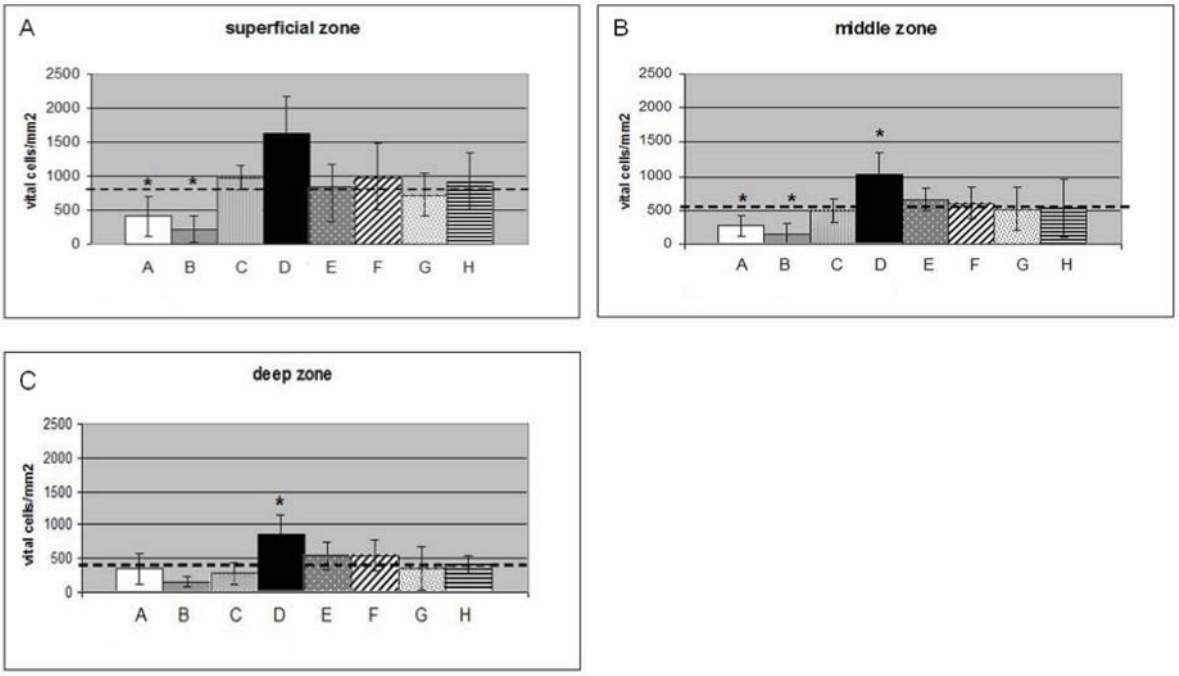

Figure 4.4. Effect of enzymatic treatment on number of vital chondrocytes in culture. Vital cell counts (mean ± SD) in wound edges of explants after 14 days in culture are presented per square millimeter. Number of vital chondrocytes in unwounded cartilage at time point zero is indicated by dashed line. Asterisk indicates statistically significant difference from unwounded cartilage at time point zero ($p \leq 0.05$). See Figure 4.1 for specification of group A-H. **A)** Superficial zone. **B)** Middle zone. **C)** Deep zone.

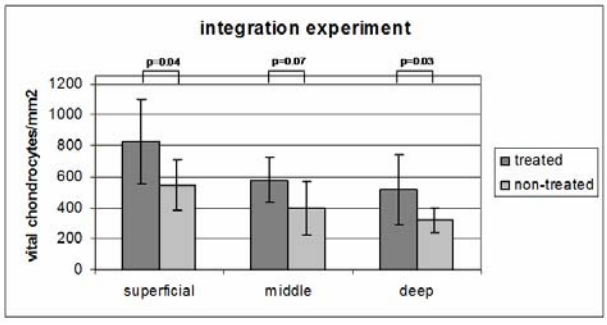

Figure 4.5. Effect of enzymatic treatment on number of vital chondrocytes in integration experiment. Number of vital chondrocytes (mean ± SD) in the wound edges of the enzyme-treated inner core compared to untreated outer wound edges of outer ring after 5 weeks of subcutaneous implantation in athymic mice.

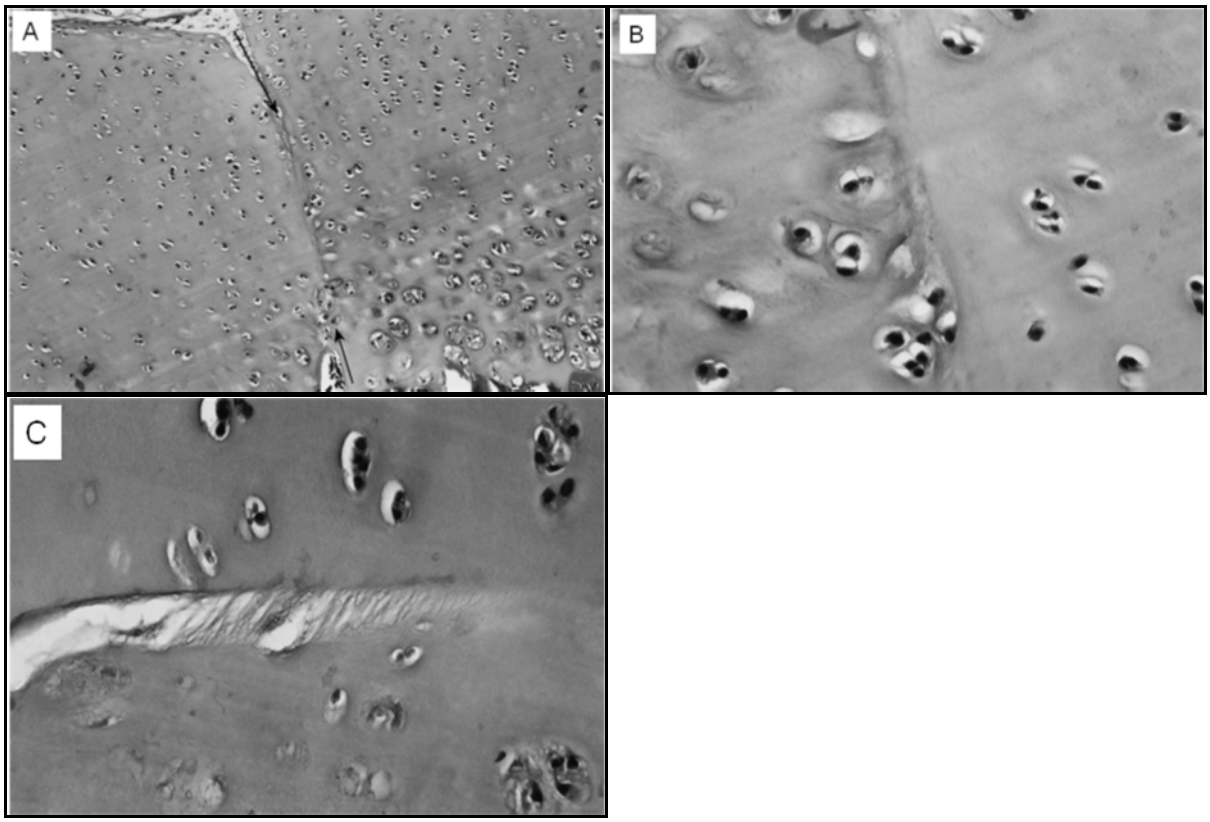

Figure 4.6. Zone of the enzyme treated cartilage in integration experiment. **A)** Chondrocyte density at the interface of treated inner core (right) and untreated outer ring (left; H&E, original x100). Integration area (arrows) shows almost 100% integration. **B, C)** Magnification of the integration area (H&E, original x400). **B)** More vital chondrocytes are visible in the enzyme-treated inner core (left) in comparison with untreated outer ring of the cartilage (right). **C)** collagen fibers are crossing over from the treated part (bottom) to untreated part (top) (*see page 113 for color figures*).

Discussion

Integration of a cartilage graft is probably hindered by a lack of matrix-producing cells in the interface region. This lack is mainly caused by chondrocyte death and the inability of chondrocytes to migrate through the extracellular matrix toward the lesion edges, in which integrative repair capacity is required. In previous studies, we demonstrated that treatment of articular explants with highly purified collagenase results in a significant increase in the number of vital chondrocytes at the wound edges in which initial cell death occurs. This “vitalisation” of wound edges, that provides vital matrix-producing cells near the cutting surface, was shown to improve integrative cartilage repair[4]. In this study, we have demonstrated that these effects can be achieved by treating for only 1 hour with a combination of highly purified collagenase and hyaluronidase. It appeared to be possible to add these two enzymes simultaneously, facilitating clinical application. Furthermore, in an in vivo integration

experiment in which only the inner core was enzyme-treated, we showed, aside from this increase in vital cell numbers, more frequent integration of the extracellular matrix with matrix fibers crossing over from the enzyme-treated part to the untreated part.

Complete repair of cartilage wounds or defects requires integration between transplanted cartilage and the surrounding cartilage at the recipient site. The fact that repair of cartilage lesions more often requires surgical removal or reshaping of cartilage, leading to associated chondrocyte death in the lesion edges would make failure of integration an inevitable phenomenon[4,6,12-14]. We propose that for cartilage integration viable chondrocytes are necessary in the wound edges. Other authors have described this important role of chondrocytes in cartilage repair. Reindel et al.[1] showed that the repair process that forms tissue at the interface between pairs of cartilage explants appeared to depend on viable cells. Peretti et al.[15] showed that chondrocytes grown on dead cartilage matrix produced new matrix that integrated individual cartilage pieces with mechanically functional tissue. In our studies, we have found a significant increase in the number of vital chondrocytes in the wound edges after enzyme treatment of cartilage in comparison to the control group.

Some studies have examined the ability of tissue-engineered cartilage to be integrated into the surrounding tissue by use of a model similar to the one we have used[16,17]. Tissue-engineered cartilage normally contains a higher chondrocyte density than native adult cartilage, and its matrix is not as tight. Obradovic et al.[16] demonstrated that immature constructs, created in 5 days, had poorer intrinsic mechanical properties than, but integrated more rapidly than, either more mature tissue-engineered constructs or native cartilage explants. Enzyme treatment with trypsin of the recipient native cartilage further enhanced the integration of 5-day constructs, but had little effect on the integration of 5-week constructs or native cartilage. Hunter et al.[17] investigated the adhesion of four different engineered cartilage constructs in an in vitro defect repair model. They used engineered tissue containing chondrocytes as inner cores and full-thickness rings of articular cartilage (8-mm diameter) of immature cattle as recipient cartilage. The constructs were cultured for 20 or 40 days and were analysed mechanically and biochemically. Differences in integration between the different engineered cartilages could not be

explained biochemically. Hunter did not look in detail at the integration area itself though. We previously demonstrated that in constructs of calf cartilage in which both the inner core and the outer ring were treated with collagenase and hyaluronidase for a total of 48 hours, cell density in the wound edges was increased and interface strength was improved[18]. This finding indicates a relation between cell density in the interface edges and the strength of bonding.

The exact mechanism by which the enzymatic treatment exerts its effects is still unclear. Application of enzymes may remove the acellular layer, uncovering an activated area of chondrocytes capable of integration. Another possible working mechanism of this enzymatic treatment may be the partial degradation of extracellular matrix surrounding the wound edge chondrocytes, stimulating chondrocyte proliferation and enabling cell migration. In our experiments we saw some chondrocyte clusters, indicating cell proliferation. In the integration experiments at the time of implantation of the inner core in the outer ring after brief enzyme treatment, we noticed a small gap between inner core and outer ring, indicating removal of the matrix at the wound edges. So, the effect of enzymatic treatment may be caused by a combination of removal of acellular matrix and stimulation of cell proliferation and migration.

The increase in amount of cells is accompanied by an increase in integration. In our in vivo integration experiment we saw that 80% of the constructs were integrated (defined by a connection between the inner core and outer ring over more than 5% of the interface area), whereas in the control group 46% of the constructs showed integration. In most cases, sections show integration on one side of the disc but not at the opposite interface area. This finding may very well be explained by the size reduction of the treated inner core. The side where the inner core made contact with the outer ring was integrated, whereas the other side was less integrated because of a small gap. This idea is supported by the percentage of the interface that was integrated. In total we found an average of 53% of the interface length to be integrated; the average for the “better integration sides” was 75%, and for the opposite “worse sides,” 26%. Because we have never noticed such a gap with previous enzyme treatments (48 hours with lower concentrations of enzymes), further optimization of enzyme conditions might be necessary for clinical application. This

study however, shows that short enzyme treatment of the graft only has the potential to improve cartilage integration.

Conclusion

We have demonstrated that brief enzymatic treatment with a combination of hyaluronidase and collagenase increases the number of vital chondrocytes in wound edges and has a positive effect on integrative cartilage repair. The integration experiment showed (new) fibers crossing over from enzyme-treated cartilage to the untreated part. This finding suggests that enzyme treatment will be favourable for integrative cartilage repair. By reducing the treatment time from 48 hours to 1 hour and treating the graft only, we are a step closer to the possible clinical application of this procedure. More studies need to be undertaken to thoroughly test the safety of enzyme treatment in order to prevent harmful effect on adjacent chondrocytes and cartilage and to further optimize its efficacy on cartilage integration in vivo.

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Chapter 5

Tracheal reconstruction: mucosal survival on porous titanium

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Abstract

Objective. To investigate whether porous titanium can provide a better support for revascularisation of a mucosal graft ideal for tracheal reconstruction. In patients with laryngotracheal stenosis or tumor, the mucosa with supporting structures can be damaged, resulting in a defect that has to be reconstructed. Autologous tissues like cartilage and mucosa have been used for reconstruction. The main problem has been incomplete mucosal re-epithelialization.

Methods. In the first experiment, porous titanium or ear cartilage was combined with mucosa and implanted subcutaneously in athymic mice for different periods of time. In the second experiment, using rabbits, surgically created defects were reconstructed with porous titanium and mucosa on a pedicled fascia flap using a two-stage procedure. The implants were analyzed with emphasis on angiogenesis and mucosal survival.

Results. Normal mucosa having a submucosal layer with vital cells was noted on top of the titanium. Multiple blood vessels were observed extending from the muscle layer through the titanium. Cytokeratin expression was detected in the suprabasal and basal layers of the mucosal epithelium. In contrast, the mucosa on cartilage showed no vital cells and no cytokeratin expression. In the rabbit experiment, all animals survived the reconstruction. The titanium was well integrated to the adjacent tracheal cartilage and surrounding tissues, supporting a fully vital mucosa.

Conclusions. Porous titanium is an inert biomaterial that provides support and allows easy revascularisation of a mucosal graft. Titanium, in combination with viable autologous tissues, is a good alternative for tracheal reconstruction.

Introduction

The surgical repair of laryngotracheal stenosis (LTS), whether it is congenital or acquired, remains one of the most challenging aspects of airway management. For subglottic and short segment stenosis, repair can be achieved with good results using cartilage interposition grafts in cricotracheal split procedures and with cricotracheal resection (CTR) followed by end-to-end anastomosis[1,2]. Resection of the trachea theoretically can be performed safely after resection of up to one-half of the trachea in adults and one-third in infants and small children. However, long segment stenosis involving more than half of the entire tracheal length and re-stenosis after an initial resection and end-to-end anastomosis, pose a therapeutic problem since further resection is usually not possible. For such cases, augmentation of the stenotic segment using repair tissues can be a valuable option.

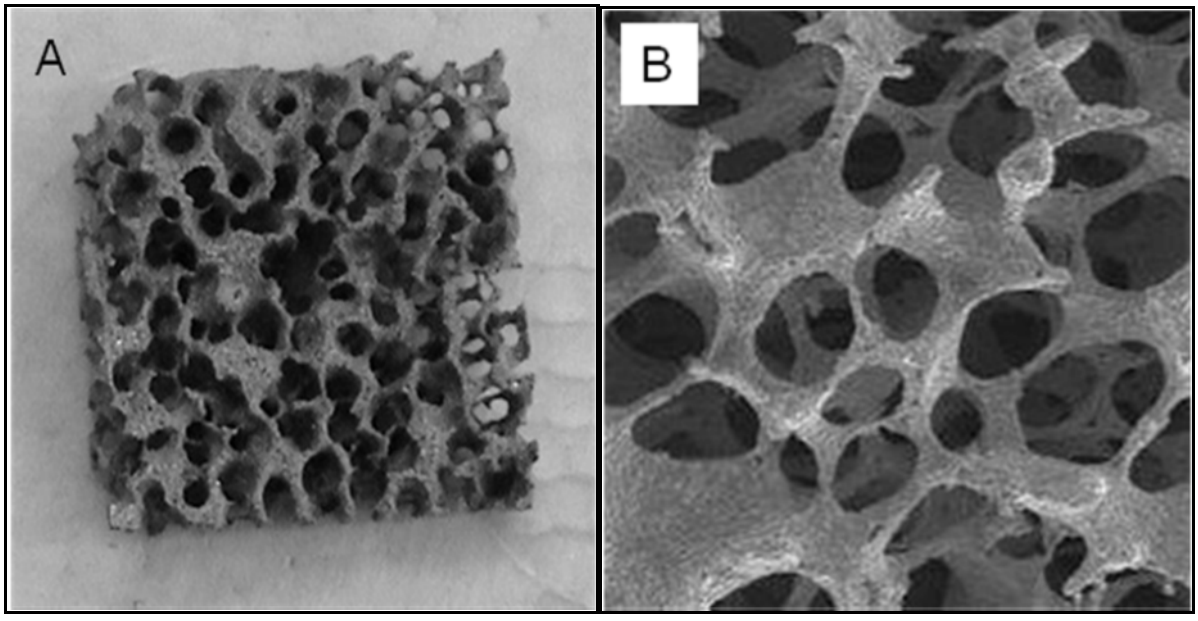

Figure 5.1. A) Titanium plate of 10 X 10 X 2 mm. with pores ranging from 400 to 700 μm and a porosity of 90%. **B)** Magnification of Figure A (original x10) (*see page 115 for color figures*).

Ideally, a tissue for laryngotracheal reconstruction would consist of a viable cartilage and respiratory mucosal lining similar to that of the native trachea. Unfortunately, there is no such composite tissue elsewhere in the body that can meet all these requirements. Most of the reconstructive tissues applied clinically lack one or both components, leading to the large variation in results. Using tissue-engineering techniques, Delaere et al.[3] created a prefabricated and prelaminated repair tissue

composed of revascularized elastic ear cartilage with buccal mucosal lining and applied it clinically with satisfactory results. A major drawback of this technique is the long waiting period that is necessary to allow revascularisation of the mucosa and remucosalization of the cartilage graft before the whole regenerated tissue can be finally transferred to the neck area. Cartilage, by nature, is avascular and possesses antiangiogenic properties, making it a less suitable support tissue for mucosal grafts. This was clearly shown by Delaere and Hardillo[4], when they reconstructed circumferential tracheal defects in rabbis with tubed auricular cartilage pre-wrapped in vascularized fascia. Despite the improvement of vascularisation around the tube, healing through remucosalization only occurred around the anastomotic regions leaving the central portions of the grafts bare and partially necrotic.

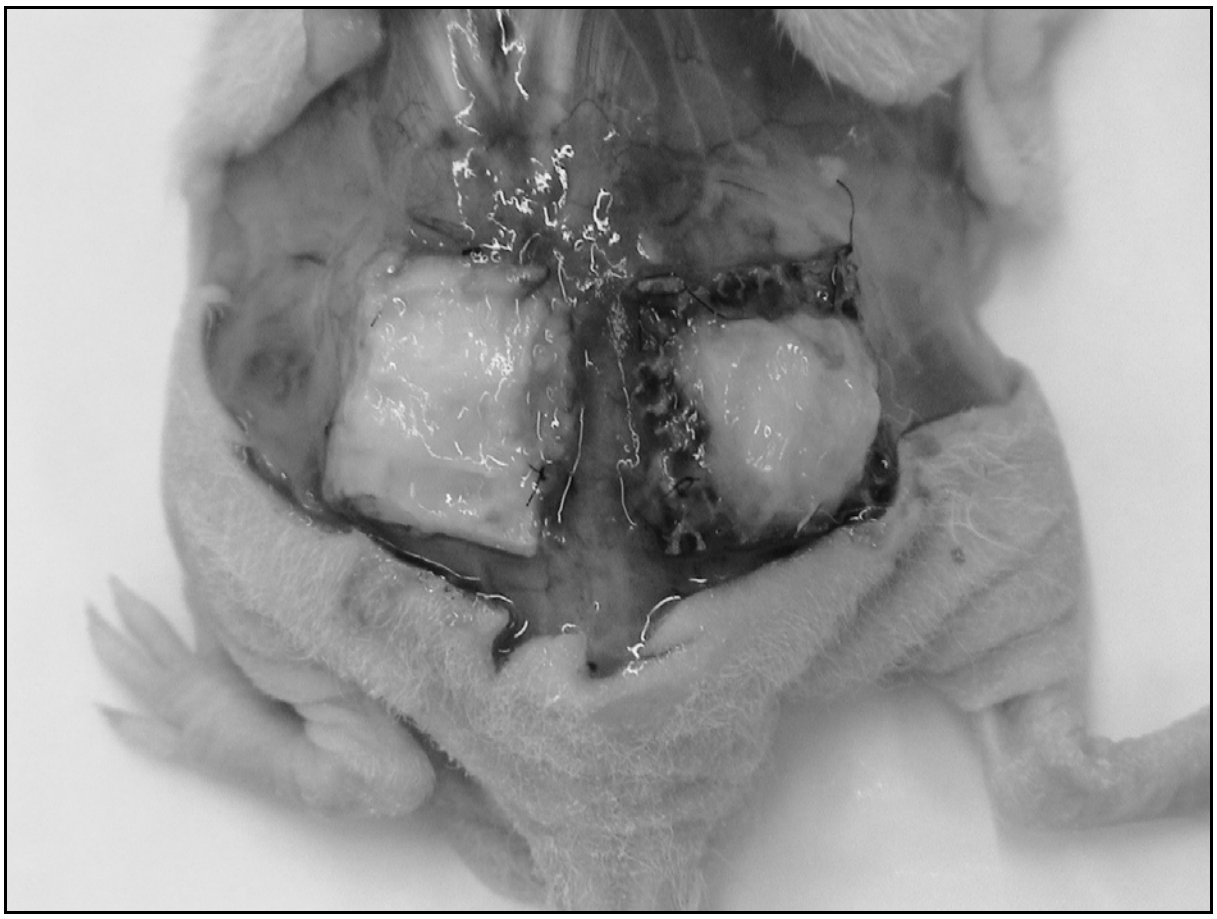

Figure 5.2. The back of an athymic mouse with on the left a cartilage piece with rabbit's buccal mucosa. On the right, a titanium plate with rabbit's buccal mucosa. This is post termination after 2 weeks of implantation (*see page 117 for color figure*).

Because of the limitations of prefabrication using autologous tissues such as the combination of cartilage and mucosa, the use of various biomaterials has been

attempted. Solid titanium, Medpor, hydroxyapatite[5-7] or tissue-engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid with or without mucosal lining [8-10] have been used in experimental settings. The main problem in all of these studies is the regeneration of the mucosal lining, as mentioned in a review by Tan et al.[11]. In cases of minor defects, re-epithelialization from the wound edges occurred, but for larger defects this process of re-epithelialization proved to be insufficient. Previous attempts to improve revascularisation of mucosal grafts using perforated cartilage proved to be futile because perforated cartilage lacked the necessary support to keep the tracheal lumen open. A possible solution to this problem is the use of stable porous biomaterials, which can allow new vessels to grow through the pores to revascularize an implanted mucosal component and thereby ensure its survival. In addition, the biomaterial of choice should be strong enough to keep the airway lumen open, and most important, the material should not evoke a foreign body reaction or inflammation.

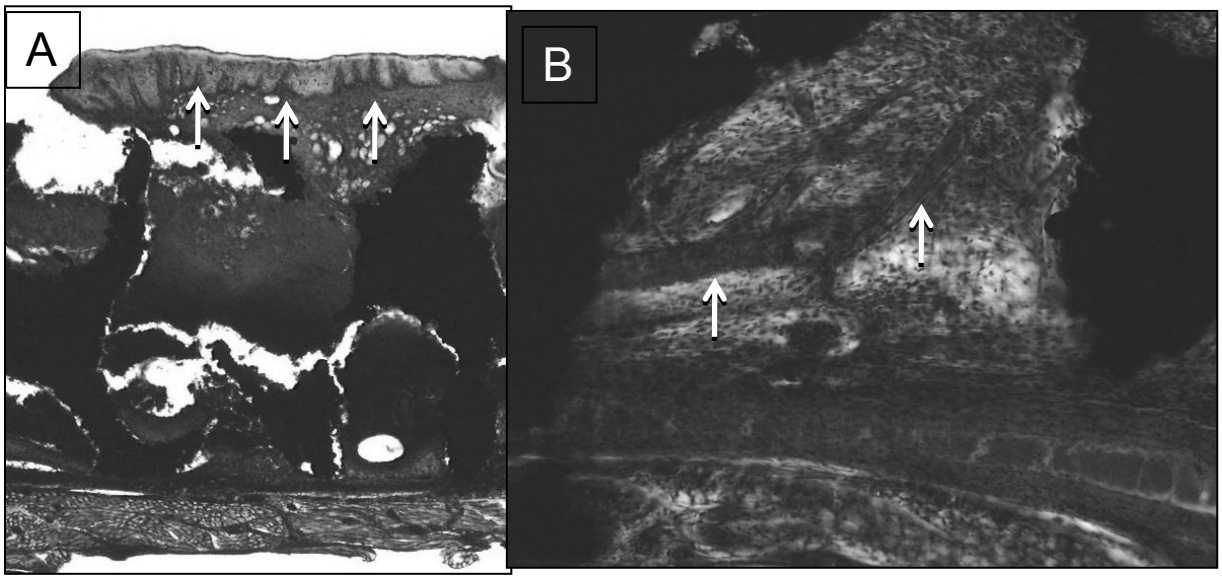

Figure 5.3. Histological section of human buccal mucosa on top of titanium after 2 weeks of implantation. **A)** Human buccal mucosa (H&E original x2.5). The black material is titanium. The mucosa is attached to the titanium. The pores of the titanium are filled with fibrous tissue and blood vessels. The white arrows point to the basal layer of the mucosa. **B)** Magnification (H&E original x10) showing the titanium filled with fibrous tissue and blood vessels (white arrows) (*see page 115 for color figures*).

These requirements are met by titanium, a material that has been used for years as an implantation material in both orthopaedics and maxillofacial reconstructive surgery. Titanium is chemically resistant to the corrosive and oxidative activities of

various agents[12]. It has also been shown to be very well tolerated by animal and human tissues, making it a suitable material for tracheal reconstruction.

We investigated whether human and rabbit buccal mucosa will survive on porous titanium in an athymic mice model and in a rabbit tracheal reconstruction model. In the athymic mouse model experiment we determined whether angiogenesis will occur through the porous titanium, allowing graft survival in comparison with cartilage grafts. In the rabbit tracheal reconstruction model experiment, we studied whether a construct of porous titanium and mucosa would be tolerated inside the trachea.

Methods

Porous titanium plates. Porous titanium implants were produced by the polymeric sponge replication method. Titanium powders (75% by weight) with a spherical shape and mean diameter of 45 μm (Bongen Titanium, Co Ltd, Xián, China) were mixed with water (18.5% by weight) to make a titanium slurry. Polyethylene glycol 4000 (Fluka Chemie GmbH, Delsendorf, Germany) and methylcellulose (MC, Fisher Scientific B.V., Landsmeer, the Netherlands) were used as binders (3.5 % by weight). One percent by weight of an alkali-free carbonic acid-based polyelectrolyte, Dolapix (Aschimmer & Schwarz GmbH, Rhein, Germany), as dispersant 1% by weight of ammonia solution (25% by weight, Merck, Darmstadt, Germany), and 1% by weight of 1-octanol (Acros Organics, Pittsburgh, Pennsylvania) were mixed in to improve the rheological property of the slurry. Porous titanium green bodies were made by impregnation of polymeric sponges (Coligen Europe B.V., Breda, the Netherlands). The polymeric sponges were thoroughly dipped into the titanium slurry. This dipping process was repeated until all the struts of the polymeric sponge were covered with slurry. Excess slurry was removed by using a roller-pressing device. After drying, the samples were heated to 500ºC in argon to burn out the foam. Finally, the porous bodies with pores ranging from 400 to 700 μm were sintered in a vacuum furnace (10^{-5} mbar) at 1250ºC for 2 hours. Porous tracheal implants with a porosity of 90% were sliced using a wire electric discharge machine[13] (Figure 5.1).

Human mucosa experiment in athymic mice. Human buccal mucosa (10 X 5 mm) of two patients undergoing an intraoral surgical procedure was harvested and used

within 1 hour with approval of the local Medical Ethics Committee (protocol No. 232.997/2003/196). The mucosa was split into two pieces of 5 X 5 mm in size, and each piece was placed on the center of a 10 X 10 X 2 mm porous titanium plate and fixed with fibrin glue (Tissucol; Baxter, Utrecht, the Netherlands). To avoid any host-graft rejection, the titanium was sutured on the back muscle of athymic mice (BALB-c nu/nu; Harlan, Horst, the Netherlands) with approval of the local animal ethical committee (Protocol No. 126-03-01). The mucosa was covered with Neuro-Patch (Braun Aesculap AG & CO KG, Tuttlingen, Germany) to prevent adhesion of the mucosa to the inner side of the skin and to limit revascularisation of the mucosa from the surrounding tissues aside from the back muscle. The mucosa from one patient was implanted for 1 week; the mucosa from the other patient was implanted for 2 weeks. Two mice were used; each animal carrying two pieces of titanium. The mice were killed respectively after 1 and 2 weeks and the titanium plates were removed including a piece of back muscle. These were embedded in plastic for histological analysis.

Rabbit mucosa experiment in athymic mice. Rabbit buccal mucosa (20 X 10 mm) was harvested from dead rabbits (acquired from another experiment) within 1 hour after euthanasia. The mucosa was split into two pieces of 10 X 10 mm and then sutured with Ethilon 6-0 on porous titanium plates or rabbit ear cartilage (from the same dead rabbits) of 10x10 mm (Figure 5.1). Both constructs were sutured subcutaneously on the back of athymic mice with approval of the local animal ethical committee (Protocol No. 126-03-01). The mucosa was covered with Neuro-Patch to prevent adhesion of the mucosa to the inner side of the skin and to limit revascularisation from the surrounding tissues aside from the back muscle. Each mouse carried a titanium construct and a cartilage construct on the back (Figure 5.2). Both constructs were covered with mucosa from the same rabbit. A total of three mice were used. The mice were killed after 1, 2, and 4 weeks respectively. Half of the mucosa was dissected carefully from the titanium or the cartilage for cytokeratin (CK) staining to evaluate the basal layer and vital keratinocytes of the epithelium. The rest of the mucosa still attached to the graft was embedded either in plastic for the titanium constructs or in paraffin for the cartilage grafts for histological analysis with emphasis on angiogenesis and mucosal survival.

Tracheal reconstruction experiment in rabbits. Three male New Zealand white rabbits (each weighing 3 kg) were used in a two-stage procedure with approval of the local animal ethical committee (Protocol No. 126-03-01). In the first stage, the rabbits were anesthetized intramuscularly with 4cm^3 of xylazine-hydrochloride, 2% (Rompun; Brussels, Belgium) and ketamine hydrochloride 10% (Ketalin; Apharmo, Duiven, the Netherlands). The left thoracic skin was incised, and a concave-shaped titanium plate of 20 X 5 mm in size together with a section of harvested full-thickness buccal mucosa of 20 X 5 mm in size was sutured with Ethilon 6-0 on the left lateral thoracic fascia flap. The titanium was covered with Neuro-Patch to prevent adhesion of the mucosa to the skin. The skin was closed in one layer using Vicryl 2-0.

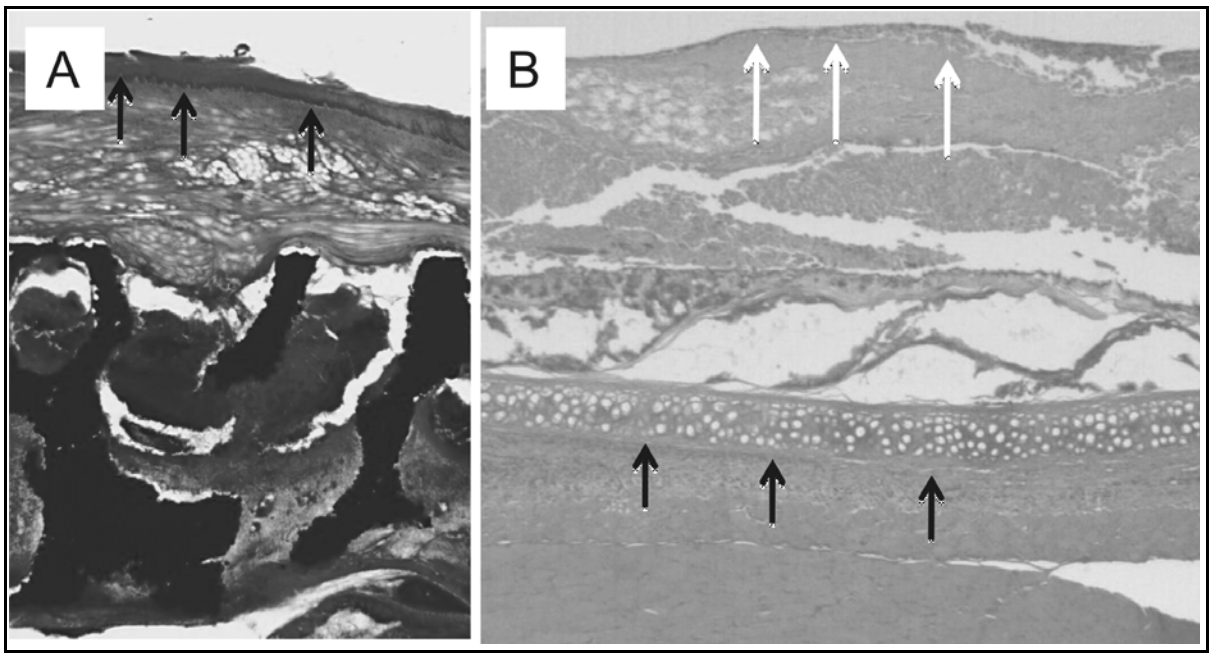

Figure 5.4. Rabbit buccal mucosa. **A)** Rabbit buccal mucosa on top of titanium (H&E magnification original x25). Note the intact basal layer as indicated by the black arrows. **B)** Rabbit buccal mucosa on top of ear cartilage (H&E magnification original x25). In the middle of the picture the cartilage is visible with its chondrocytes (black arrows). There is no connection between the mucosa and the cartilage. The basal layer of the mucosa is not visible (white arrows) (*see page 117 for color figures*).

In the second stage, 2 weeks after implantation, the skin was incised, and the left thoracic fascia flap was isolated on the lateral thoracic vessels and rotated under the skin into the neck region of the rabbit. Using a midline incision, the anterior cervical trachea was dissected and a defect of 20 X 5 mm was created in the anterior cricoid and the first three tracheal rings. The defect was one-third of the total diameter. The mucosa/titanium attached to the fascia flap was then sutured with Ethilon 6-0 into the defect with the buccal mucosa facing the tracheal lumen. The skin was closed in

three layers. After 6 weeks, the rabbits were killed with an overdose of pentobarbital, and the cricotracheal complex was dissected out. The trachea was opened posteriorly in a vertical line and photographed. The trachea was then embedded in plastic for histological analysis.

Histological analyses. In the human mucosa-athymic mice experiment, all pieces were embedded in plastic and sections of 50 µm in thickness were made using a diamond saw (Leica SP1600; Leica AG, Glattbrugg, Switzerland) and stained with hematoxilin and eosin.

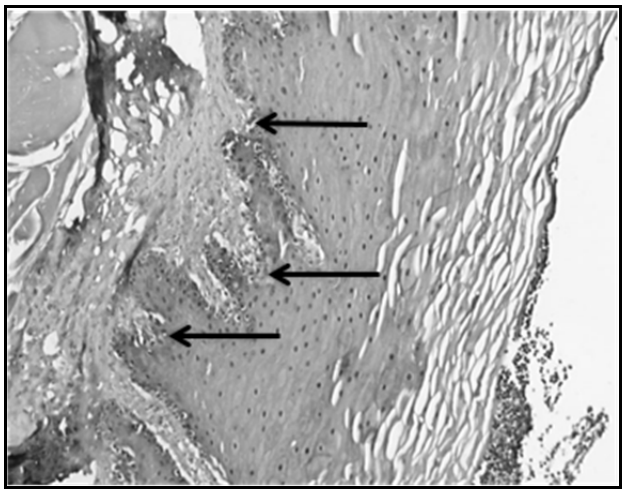

Figure 5.5. Cytokeratin staining (CK13) on rabbits buccal mucosa after 2 weeks of implantation with a clear basal membrane (black arrows) and suprabasal keratinocytes of vital mucosa (original magnification x100, visualization with New Fuchsin, counterstain with Gill's haematoxylin) (*see page 117 for color figure*).

In the rabbit mucosa-athymic mice experiment, half of the mucosa was dissected carefully from the titanium or the cartilage and was snap frozen in liquid nitrogen. Six micron sections were cut for CK staining (see next subsection) to evaluate the basal layer and vital keratinocytes of the epithelium. The rest of the mucosa still attached to the graft was embedded in either plastic for the titanium constructs or in paraffin for the cartilage grafts. For the titanium, 50 µm sections were made using a diamond saw (Leica SP1600). For the cartilage, 6 µm sections were made using a microtome (Leica RM 2135). Both the plastic and the paraffin sections were stained with hematoxilin and eosin. In the tracheal reconstruction experiment, 50 µm sections were made using a diamond saw (Leica SP1600) and stained with hematoxilin and eosin.

Immunohistochemical analyses in the athymic mice. Cytokeratin expression of the mucosa was evaluated using antibodies against CK10 (Euro-diagnostica, Arnhem, the Netherlands), CK13 (Euro-diagnostica), CK14 (Immunotech, Marseille, France) and CK16 (Nove Castra, Newcastle, England). CK10 and CK13 are markers for the suprabasal layers of stratified mucosa, CK14 is a marker for the basal layers of stratified epithelia, and CK16 is a marker for suprabasal cells of hyperproliferative squamous epithelia. Positive controls of these CK's were performed on a piece of rabbit buccal mucosa.

All samples were fixed in formalin, processed in paraffin wax, and sectioned at a thickness of 6 µm. To stain mouse sections with a mouse antibody, the primary and secondary antibody (alkaline phosphatase conjugated goat anti-mouse IgG, Immunotech) had to be prelinked overnight[14]. For CK10, CK14 and CK16 sections were postfixed in formaldehyde, 4% for 10 minutes after deparaffinization and antigen retrieval was performed out by immersing sections in sodium citrate buffer, 0.01 M (pH 6.0) and keeping them at boiling temperature for 10 minutes using a microwave oven. Cytokeratine 10 staining required antigen retrieval with trypsin, 0.075%, in phosphate-buffererd saline for 30 minutes at 37°C. After retrieval, nonspecific reactions were blocked using 10% normal goat serum (Sigma-Aldrich, St Louis, Missouri) for 30 minutes followed by incubation with the pre-linked antibodies for 1 hour and alkaline phosphatase (alkaline phosphatase anti-alkaline phosphatase [ASPAAP], DakoCytomation, Glostrup, Denmark) for 30 minutes. Visualisation was performed with an alkaline phosphatase substrate New Fuchsin (Chroma, Köngen, Germany). Sections were counterstained with Gill haematoxylin (Sigma-Aldrich) for 1 minute. The negative control was performed using a nonspecific mouse IgG1 (DakoCytomation, X0931) also prelinked with the alkaline phosphatase conjugated goat antimouse IgG[15].

Results

Viability of human mucosal grafts on titanium. Vital mucosa was seen attached to the titanium (Figure 5.3A). On histological evaluation, normal mucosa with a submucosal layer was visible with vital cells. Multiple blood vessels were observed growing from the muscle layer into the titanium in the direction of the mucosa (Figure 5.3B). This

process was seen as early as 1 week with more pronounced vessel formation after 2 weeks.

Viability and morphology of rabbit mucosal grafts on titanium versus cartilage. Vital buccal mucosa, adherent on the titanium was observed in all sections (Figure 5.4A). The mucosa had a normal structure consisting of epithelial cells and a submucosal layer. As early as 1 week post implantation, new blood vessels were already visible, originating from the back muscle of the mouse and growing into the titanium up to the submucosal layer.

A normal basal layer was present in all three mice (1, 2 and 4 weeks after implantation) as shown in Figure 5.5. The suprabasal layers were more pronounced after 1 and 2 weeks. Cytokeratin expression was detected in the suprabasal (CK10 and CK13) and basal (CK14) layers of the mucosa on the titanium. No expression of CK16 was seen.

The mucosa on the cartilage showed varying degrees of necrosis and no revascularisation. The mucosal layer was not integrated with the ear cartilage (Figure 5.4B). No CK expression was detected in this material.

Performance of titanium-mucosal graft construct in tracheal reconstruction. All three rabbits survived the two operations until the end of the experiment, 6 weeks after tracheal reconstruction. In two rabbits the titanium was covered with mucosa and the airway lumen was open (Figure 5.6A and B). Granulation tissue was seen in only one of the suture points in one rabbit. The third rabbit became dyspnoeic at the end of the experiment. On dissection of the cricotracheal complex, a segment of the titanium was noted to have shifted inside the trachea.

Histological evaluation of the mucosa and the titanium showed revascularisation of the mucosa through the titanium and a vital mucosa (Figure 5.6C). In one rabbit some granulation tissue was found around a suture, as noted on macroscopic inspection. Otherwise, no signs of inflammation or foreign body reaction were visible. The constructs were well integrated with the adjacent cartilage and soft tissues (Figure 5.6D).

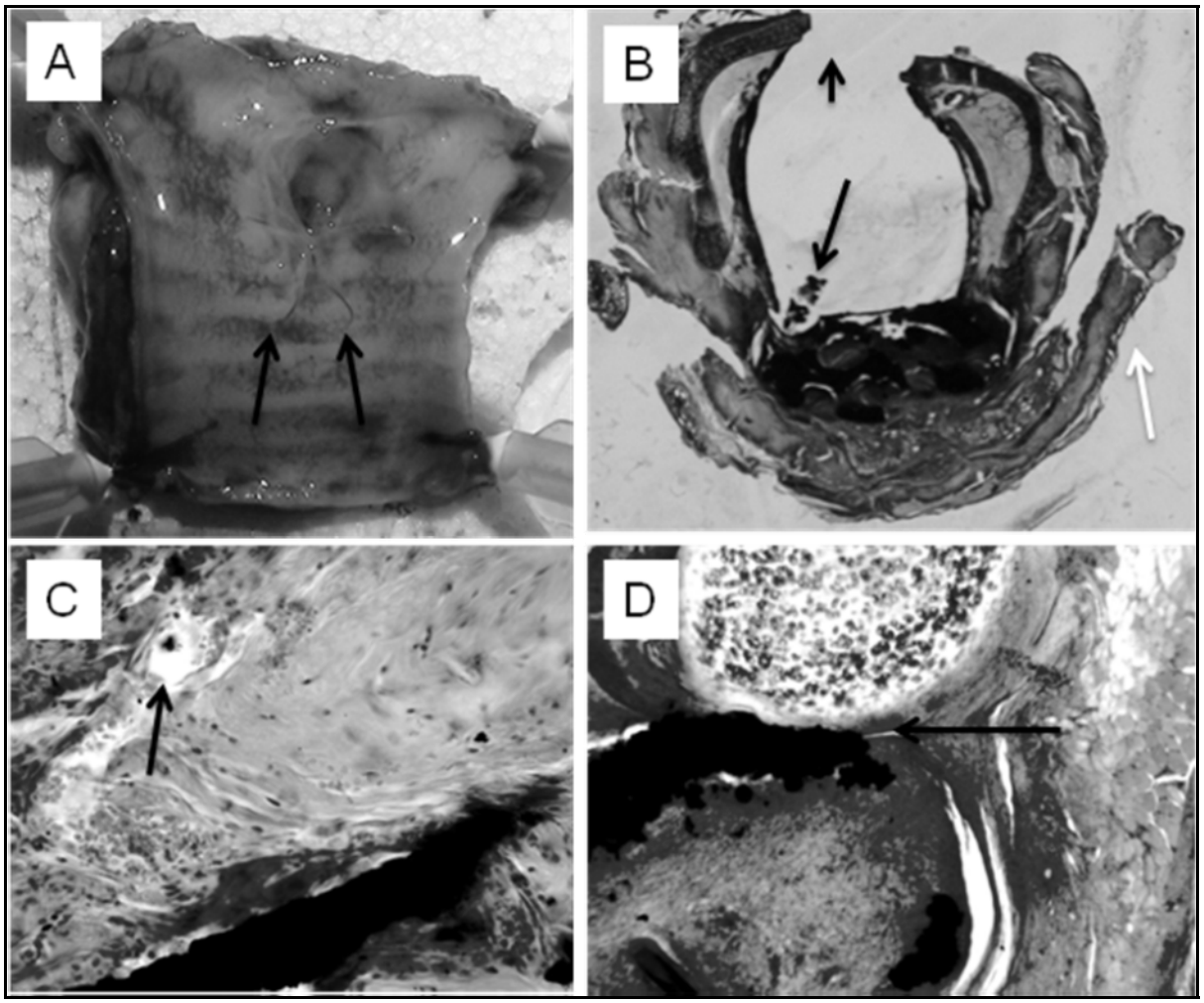

Figure 5.6. Cricotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. **A)** The titanium is visible under the mucosa with stitches (black arrows). The titanium is covered with a thin layer of mucosa. **B)** Histological cross-section through the trachea (H&E). The short black arrow indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. A thin layer of mucosa is covering the titanium. Some granulation tissue is present on the left side of the titanium just inside the tracheal lumen (long black arrow). **C)** The pores in the titanium (H&E, original magnification x200). Notice the presence of fibrous tissue filling the pores of the titanium. The black arrow is pointing at a bloodvessel. **D)** The integration area between the cartilage and titanium (H&E original magnification (x100). Notice a tight connection is present between the titanium and the cartilage (black arrow) (*see page 119 for color figures*).

Discussion

Surgical repair of the trachea remains a therapeutic challenge. Although long-segment stenosis is a rare entity, acquired stenosis is a growing problem owing to advances in medical care that have result in increased numbers of critically ill patients requiring chronic mechanical ventilation[16].

At present, subglottic and short segment stenosis at present are mainly repaired by cartilage interposition grafts in cricotracheal split procedures and with CTR followed by end-to-end anastomosis[17]. Long-segment stenosis involving more than half of the entire tracheal length and re-stenosis after an initial resection and end-to-end anastomosis, continues to be a therapeutic problem because further resection is usually not possible.

There are a couple of clinical case reports in which a neo-trachea was formed using autologous costal cartilage or seeded chondrocytes in a marlex mesh tube with palatal mucosal graft[18,19]. All patients survived the procedures, but the disadvantage was the need for two or three steps, which were necessary to create the neo-trachea. Another study described a series of 112 patients in which preserved allografts from dead people were used for tracheal reconstruction in long segment stenosis[20]. However, they reconstructed only a part of the trachea, and, more over, the use of homografts in this time of Creutzfeld Jacob and other possible diseases would not be the first choice of therapy.

To improve reconstruction of the trachea, several experimental approaches have been followed in animal models. As mentioned in our introduction, autologous cartilage with laryngotracheal frameworks from solid titanium, Medpor or hydroxyapatite[5,7], tissue-engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid with or without mucosal lining[8-10] and prefabricated tubes with cartilage strips and a vascularized muscle or fascia[21] have all been used. They all had the same problem, that is, the restoration of the mucosal lining through re-epithelialization. This occurred from the wound edges in small defects, but for larger defects this process of re-epithelialization was not sufficient. The key to the problem is speeding up the process of revascularisation. This can be achieved with the use of porous materials, in which new vessels can grow through the pores to reach the epithelializing surface. Porous hydroxyapatite was used in rabbits by Triglia et al.[22]. Their main problem was the fixation of the hydroxyapatite to the surrounding tissue. Schultz et al.[23] implanted porous titanium tracheal prostheses into 17 rats. Six rats died, five of them because of displacement of the titanium or sealing of the prosthesis from overgrowth of granulation tissue. The titanium, however, was well-tolerated by the surrounding tissue as seen on histological analysis.

An optimal tissue for tracheal reconstruction should have three different components: a support tissue to keep the lumen open, a good epithelial lining, preferably consisting of mucosa, and a vascular supply[3]. The results of our study showed that these criteria can be met by using porous titanium in combination with autologous tissues. Titanium is sturdy enough to keep the lumen open, and the porous matrix offers the opportunity for blood vessels to grow into the scaffold. This allows re-epithelialization and survival of a mucosal graft. An angiogenic effect of titanium clips in rats has been described by Foschi et al.[24]. They observed new blood vessels in the mesentery of rats after creating a wound which was closed with titanium clips. Titanium has also been described to be an ideal biomaterial in a septic environment like the trachea, and our rabbit study confirmed this. No inflammatory response or foreign body reactions were observed after 6 weeks.

To our knowledge, this is the first study that used porous titanium in combination with mucosal grafts, and it clearly showed histological evidence of revascularisation of the mucosal graft. After 1 week, new blood vessels were observed growing from the underlying tissue through the titanium scaffold. The presence of the Neuro-patch eliminated possible revascularisation of the mucosa coming from the surrounding tissues other than the back muscle. The importance of this revascularisation was also highlighted by the observation that the mucosal grafts in combination with cartilage grafts appeared non-vital.

Mucosal grafts can be fixed on the scaffold using stitches or glue. In our first study using human mucosa, human fibrin glue (Tissuecol) was used. Glue is easy to apply, and the mucosa remained well fixed on the titanium, even 4 weeks after implantation. Stitching the mucosa might last longer, but some factors have to be taken into consideration, namely, that the tissue might be harmed by the stitches, it might partly detach and, depending on the material used, granulation tissue may form around the stitches. For the rabbit experiments, we did choose to use stitches to avoid possible reactions against the human fibrin glue. In theory, however, fibrin glue can offer an advantage by stimulating angiogenesis[25].

Conclusion:

Our experiments showed that reconstruction of a rabbit laryngeal defect using composites of porous titanium and mucosal grafts is a promising technique. Porous titanium is an inert biomaterial that allows easy penetration of blood vessels and survival of the mucosa. The morphologic characteristics of the mucosa were preserved, indicating a functional tissue. Future experiments have to fine-tune the use of such composite grafts for clinical use, especially when larger and circular defects have to be reconstructed.

Acknowledgments

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Chapter 6

Laryngotracheal reconstruction in rabbits with porous titanium: *Are vascular carriers and mucosal grafts really necessary?*

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Abstract

Objective. Laryngotracheal reconstruction requires a supportive structure with a mucosal lining which needs a vascular supply in order to regenerate properly. We investigated the necessity of a vascular carrier and mucosal graft when using porous titanium for laryngotracheal reconstruction.

Methods. Surgical defects of the laryngotracheal complex in 22 rabbits were reconstructed with (I) porous titanium implanted on a vascularized fascia combined with a buccal mucosal graft (first stage) before transposing to the neck area (second stage); (II) porous titanium implanted on a vascularized fascia (first stage) combined with a mucosal graft (second stage); (III) porous titanium on a pedicled fascia flap; and (IV) porous titanium alone.

Results. The grafts were tolerated well. Re-epithelialization occurred in all groups. Normal mucosa with a submucosal layer containing vital cells was noted on the titanium. Blood vessels were grown in the pores of the titanium scaffold to supply the overlying mucosa. The scaffold was well integrated in the adjacent tracheal cartilage and surrounding tissues, except in the two cases that showed titanium displacement. Inflammation and granulation formation were seen in most rabbits in groups I and II, initiated probably by the use of buccal mucosal grafts.

Conclusion. Reconstruction of a rabbit's trachea using composites of porous titanium, mucosal grafts and a fascia flap is feasible. Titanium seems to meet the requirements needed for closing a small defect of the tracheal wall and allows for re-epithelialization. For larger defects a vascular carrier with a mucosal graft is probably indispensable to ensure the process of re-epithelialization.

Introduction

Creating a new functional laryngotracheal tissue is the ultimate goal for patients with laryngotracheal stenosis (LTS) and with re-stenosis after failed resections and anastomoses, and after tracheal resection for tumours of the laryngotracheal complex and the surrounding tissues.

The limitations of composite autologous tissues, such as cartilage and mucosa, stimulated the introduction of various biomaterials. Solid titanium, Medpor, hydroxyapatite[1-3] or tissue-engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid matrices have been used in experimental settings[4-6]. The main problem in all these studies is the regeneration of the mucosal lining, as mentioned in a review by Tan et al.[7]. In cases of minor defects, re-epithelialization from the wound edges occurred successfully, but for larger defects this process of re-epithelialization proved to be insufficient, such that mucosal grafts need to be added to the constructs. These mucosal grafts, on the other hand require a good vascular bed or matrix in order to ensure its survival.

In humans, a neo-trachea was formed using autologous costal cartilage or seeded chondrocytes in a marlex mesh tube with palatal mucosal graft[8,9]. All patients survived these procedures, but the disadvantage was the need for staged surgery, which is necessary to create the neo-trachea. Recently, Machhianrini et al.[10] described a clinical case where they used a homograft trachea bioengineered with autologous stem cells with success. This procedure is technically challenging and not suitable for large-scale application.

In the experimental setting in rabbits, a neo-trachea was created from a cartilage tube wrapped with a fascia flap to improve vascularisation around the tube. Healing through remucosalization only occurred around the anastomotic regions, leaving the central portions of the grafts bare and partially necrotic[11]. Although the use of a cartilage graft to support the structure would make sense, cartilage does not support vessel ingrowth well and therefore is not a suitable material for mucosa regeneration. The process of re-epithelialization from the wound edges and revascularisation of mucosal grafts can be improved by the use of a stable porous biomaterial. This biomaterial should allow new vessels to grow through the pores, thereby creating a

vascular bed to support re-epithelialization and survival of the implanted mucosal component. The material should not evoke inflammation or a foreign body reaction.

Titanium is a material that has been used for years as implantation material in a variety of anatomical sites. Porous titanium is a relatively new product that allows blood vessels to grow through its pores[12]. Previously, we demonstrated that mucosal grafts can survive on porous titanium plates implanted on the back of athymic mouse[13]. Multiple blood vessels extending from the muscle layer through the porous titanium were observed as early as 1 week after implantation. Using these combined constructs of a porous titanium plate with a mucosal graft attached to a vascularized fascia, we successfully reconstructed surgically-created laryngotracheal defects in rabbits [13].

The aim of this study is to investigate the necessity of a vascular carrier when using a porous titanium implant with or without a mucosal graft component in the reconstruction of full-thickness (cartilage and mucosa) laryngotracheal defects in rabbits. The general question is whether the rabbits can survive such reconstructions for a specific period of time and more specific questions are: a) does a fascia flap in combination with a mucosal graft and titanium lead to better results, b) is it necessary to implant the mucosal graft on the titanium in a first stage to allow vascularisation, or can we use the mucosal graft directly after harvesting; and c) is it necessary to use a fascia flap as a vascular supply?

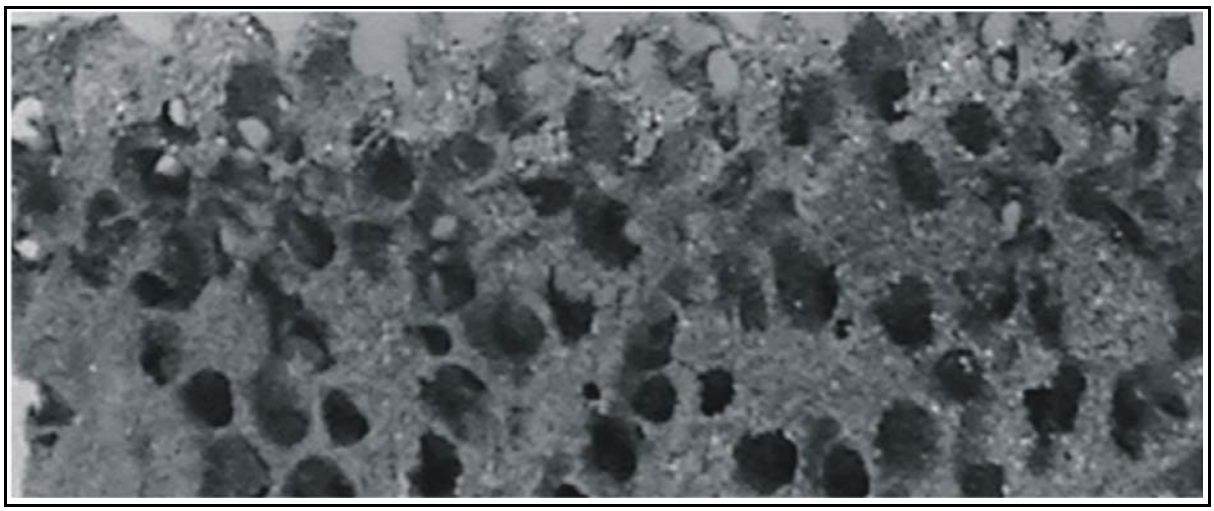

Figure 6.1. Curved titanium plate of 20 X 5 mm and a thickness of 2 mm, with pores in the range 400-700 µm and a porosity of 90% (*see page 121 for color figure*).

Methods

Porous titanium plates. Porous titanium plates with a porosity of 90% were sliced using a wire electric discharge machine in curved plates of 20 X 5 mm (Figure 6.1). Porous titanium implants were produced by the polymeric sponge replication method as described in our previous article[13].

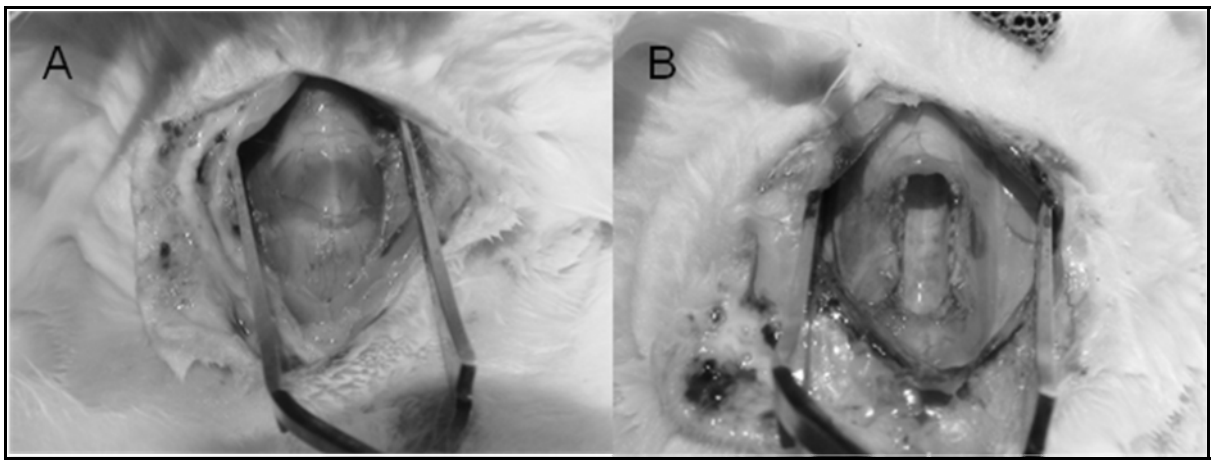

Figure 6.2. Cervical area of the rabbit exposed **(A)** before and **(B)** after creation of the full-thickness defect (through cartilage and mucosa) measuring 20 X 5 mm in the anterior cricoid and the first three tracheal rings. The defect is one-third of the total circumference. The head is downwards in this image (*see page127 for color figures*).

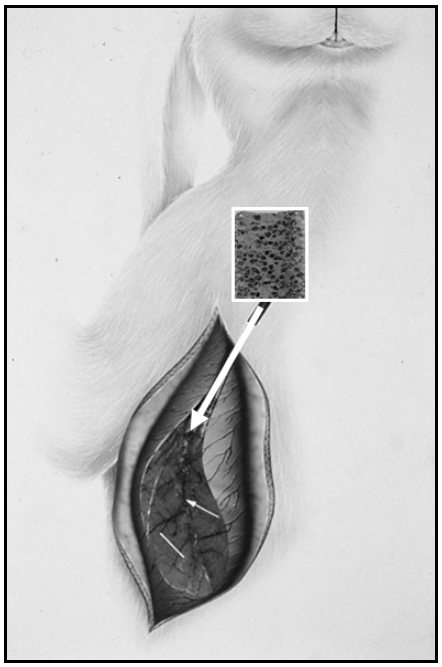

Figure 6.3. Schematic drawing of a rabbit with the titanium plate placed on the left lateral thoracic fascia flap (*see page 119 for color figure*).

Surgical procedures. Male New Zealand white rabbits (each weighing 3 kg) were used with approval of the local animal ethical committee (Protocol No. 126-03-01). For each of the four groups six rabbits were planned. Initially, three rabbits were operated on in each group. When there were no signs of dyspnea or stridor, the remaining three rabbits in each group were operated.

The rabbits were anesthetized intramuscularly with 4cc of xylazine-hydrochloride, 2% (Rompun; Brussels, Belgium) and ketamine hydrochloride, 10% (Ketalin; Apharmo, Duiven, the Netherlands). The anterior cervical trachea was dissected using a midline vertical neck incision. (Figure 6.2A)

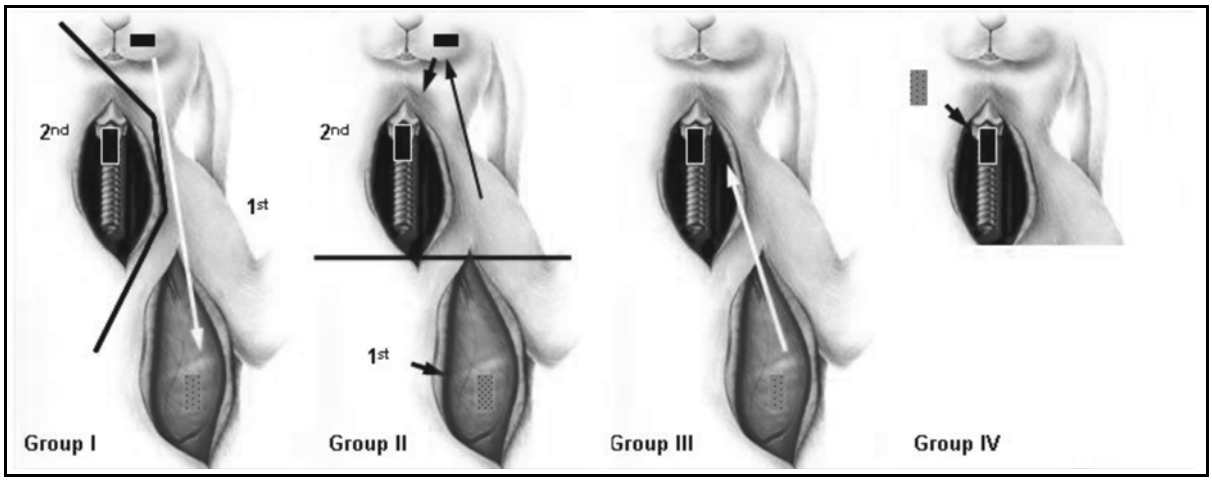

Figure 6.4. Schematic drawing of the four different groups. Groups I and II are operated on using a two-stage procedure; groups III and IV are operated on using a one-stage procedure (*see page 123 for color figure*).

Group I: porous titanium initially implanted on a vascularized fascia and combined with buccal mucosal graft (first stage) before transposing to the neck area (second stage)

In the first stage, the left thoracic skin was incised and a concave shaped titanium plate of 20 X 5 mm, together with harvested full-thickness buccal mucosa of 20 X 5 mm was sutured with Ethilon 6-0 on the left lateral thoracic fascia. The titanium was covered with Neuro-Patch (Medcompare, USA) to prevent adhesion of the mucosa to the skin. The skin was closed in one layer using Vicryl 2-0.

In the second stage, 2 weeks later, the skin was incised and the left thoracic fascia flap was isolated on the lateral thoracic vessels and rotated under the skin into the neck region of the rabbit. Using a midline incision, the anterior cervical trachea was dissected and a defect of 20 X 5 mm was created in the anterior cricoid and the first

three tracheal rings. The defect was one-third of the total circumference (Figure 6.2B). The mucosa-titanium construct attached to the fascia flap was sutured with Ethilon 6-0 into the defect, with the buccal mucosa facing the tracheal lumen. The skin was closed in three layers.

In this group only three of the six rabbits were operated because one of the rabbits became dyspnoeic at the end of the experiment. Upon dissection of the cricotracheal complex, a segment of the titanium was noted to have shifted inside the trachea.

Group II: porous titanium implanted on a vascularized fascia (first stage) and combined with a buccal mucosal graft (second stage).

In the first stage, the left thoracic skin was incised and a concave shaped titanium plate of 20 X 5 mm was sutured with Ethilon 6-0 on the left lateral thoracic fascia. The titanium was covered with Neuro-Patch to prevent adhesion of the titanium to the skin. The skin was closed in one layer using Vicryl 2-0.

In the second stage, 2 weeks later, the skin was incised and the left thoracic fascia flap was isolated on the lateral thoracic vessels and rotated under the skin into the neck region of the rabbit. Harvested full-thickness buccal mucosa of 20 X 5 mm was sutured on the titanium. Using a midline incision, the anterior cervical trachea was dissected and a full-thickness defect of 20 X 5 mm was created in the anterior cricoid and the first three tracheal rings. The defect was one-third of the total circumference. The mucosa-titanium construct attached to the fascia flap was sutured with Ethilon 6-0 into the defect with the buccal mucosa facing the tracheal lumen. The skin was closed in three layers.

Group III: porous titanium with vascularized fascia (single stage procedure).

The left thoracic skin was incised and a concave shaped titanium plate measuring 20 X 5 mm was sutured with Ethilon 6-0 on the left lateral thoracic fascia (Figure 6.3). A flap was then isolated on the lateral thoracic vessels and rotated under the skin to the neck region. Using a midline incision, the anterior cervical trachea was dissected and a full-thickness defect (cartilage and mucosa) of 20 X 5 mm was created in the anterior cricoid and the first three tracheal rings. The defect was one-third of the total circumference. The titanium attached to the fascia flap was sutured with Ethilon 6-0 into the defect with the titanium facing the tracheal lumen. The skin of the neck region

was closed in three layers, the skin of the thorax was closed in one layer using Vicryl 2-0.

Group IV: porous titanium exclusively.

A full-thickness defect (through cartilage and mucosa) measuring 20 X 5 mm was created in the anterior cricoid and the first three tracheal rings. The defect was one-third of the total circumference. A concave shaped titanium plate of 20 X 5 mm was sutured with Ethilon 6-0 to the defect. The skin was closed in three layers.

Figure 6.4 shows the four groups in a schematic overview.

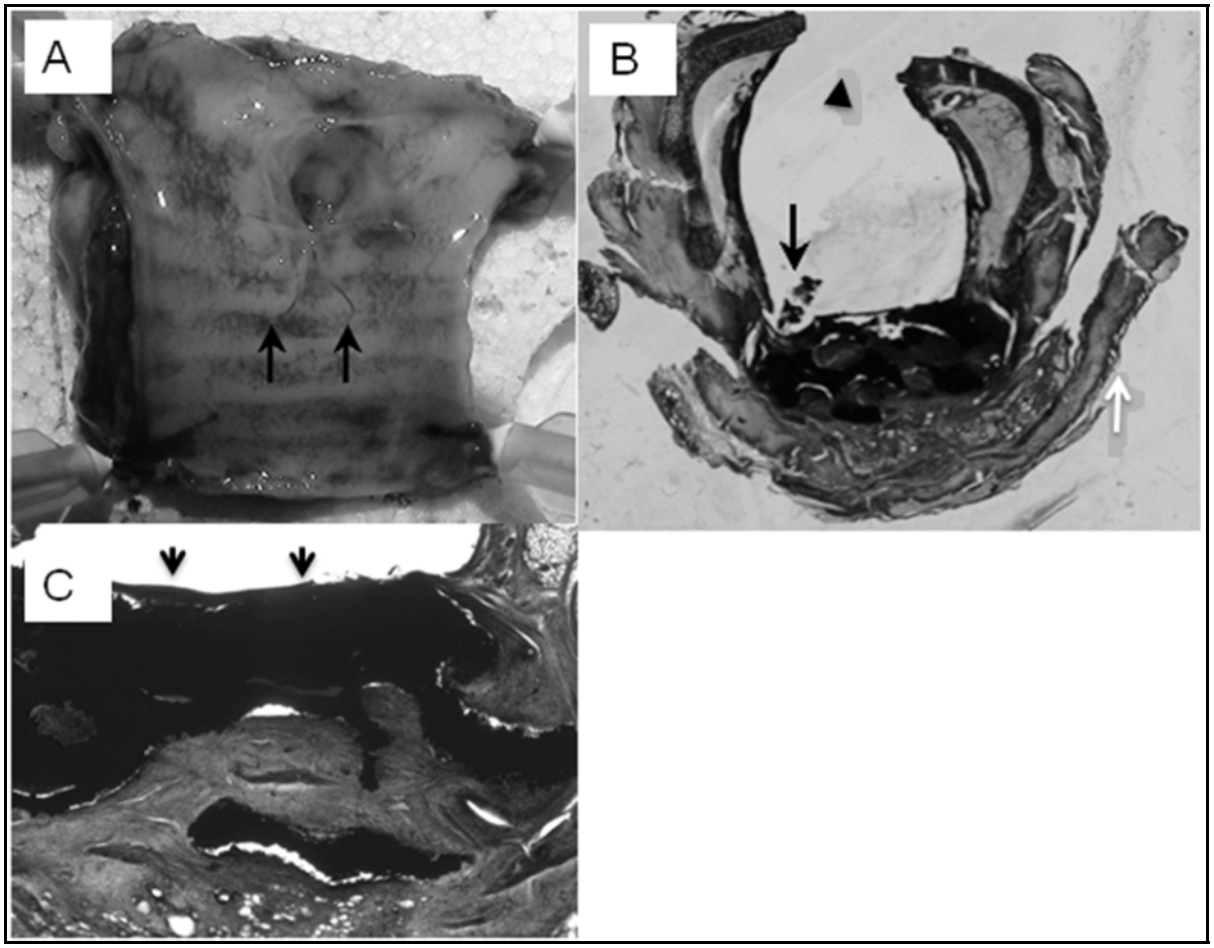

Figure 6.5. Group I: porous titanium initially implanted on a vascularized fascia and combined with buccal mucosal graft (first stage) before transposing to the neck area (second stage). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa (black arrows). **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. The black arrow is pointing at the integration area, with some granulation tissue on the mucosa visible. **C)** Magnification (H&E, original x100,) of the integration area between the native tracheal mucosa and the mucosal graft. There is a thin mucosal layer visible covering the titanium (black arrows) *(see page 121 for color figures).*

Macroscopic evaluation. After 6 weeks, the rabbits were killed with an overdose of pentobarbital and the laryngotracheal complex was dissected. Before termination, Groups I, II, and III underwent injection of the lateral thoracic artery with blue Microfil silicon dye (Canton Bio-Medical Products Inc., Boulder, Colo.) to assess the degree of revascularisation of the porous titanium and the mucosal graft component. The trachea was opened posteriorly in a vertical line and photographed. The trachea was then embedded in plastic for histological analysis.

The reconstructed defects were evaluated in terms of mucosal covering over the titanium, presence of granulation tissue formation (signs of inflammation) between the titanium and native trachea, presence of titanium displacement and the connection between the titanium and cartilage.

Microscopic evaluation. All reconstructed laryngotracheal complexes were fixed in formaldehyde and embedded in plastic and sections of 50 µm in thickness were made using a diamond saw (Leica SP1600; Leica AG, Glattbrugg, Switzerland) and stained with hematoxylin and eosin to evaluate integration of the graft and the surrounding tissues as well as vascularisation and viability of the mucosa. The mucosa was defined as vital when a normal pseudostratified ciliated columnar epithelium was identified with a basement membrane and a submucosal layer.

Results

Group I: porous titanium initially implanted on a vascularized fascia and combined with buccal mucosal graft (first stage) before transposing to the neck area (second stage).

The first three rabbits survived the two operations until the end of the experiment, 6 weeks after tracheal reconstruction. In two rabbits the titanium was covered with mucosa and the airway lumen was open (Figure 6.5A). Granulation tissue was seen in one of the suture sides in one rabbit. The third rabbit became dyspnoeic at the end of the experiment. On dissection of the cricotracheal complex, a segment of the titanium was noted to have shifted into the lumen of the trachea. Because of this we decided not to operate on the other three rabbits.

Histological evaluation of the mucosa and the titanium showed revascularisation of the mucosa through the titanium, and a vital mucosa with normal aspects covering the titanium. In one rabbit some granulation tissue was found around a suture as noted on macroscopic inspection (Figure 6.5B); otherwise, no signs of inflammation or foreign body reaction were visible. The constructs were well integrated with the adjacent cartilage and soft tissues (Figure 6.5C).

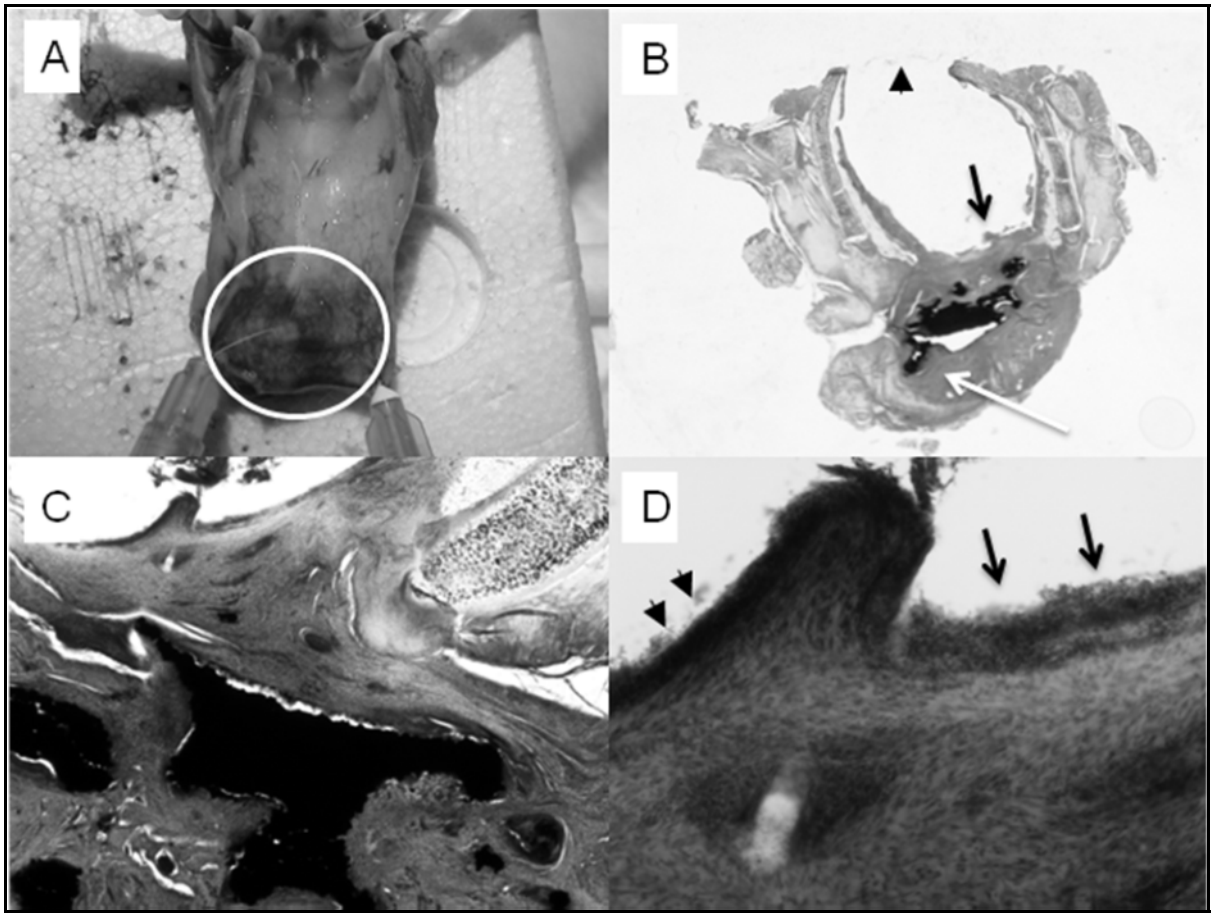

Figure 6.6. Group II: porous titanium implanted on a vascularized fascia (first stage) and combined with a buccal mucosal graft (second stage). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa. Notice the dark color of the Microfil injection (circled) showing revascularisation of the cervical trachea. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. The black arrow is pointing at the integration area, with some granulation visible on top of the mucosa. **C)** Magnification (H&E, original x100) of the integration area between the native tracheal mucosa and the mucosal graft. There are clear mucosal and submucosal layers visible, covering the titanium. **D)** Magnification (H&E, original x400) of the integration area between the native tracheal mucosa (black arrows) and the buccal mucosal graft (arrow points) (*see page 123 for color figures*).

Group II: porous titanium implanted on a vascularized fascia (first stage) and combined with a buccal mucosal graft (second stage).

The first three rabbits survived the experiment. In the second three rabbits, one rabbit was found dead after 3 days. On autopsy, the titanium was found to have been dislodged outside the tracheal lumen. Another rabbit was taken out of the experiment because of dyspnea and failure to eat. In this rabbit the titanium was found to have been dislodged into the lumen of the trachea. In one rabbit the titanium was slightly displaced into the lumen. The titanium was covered with mucosa. In two rabbits there was some granulation tissue around the sutures between the titanium and native trachea (Figure 6.6B and C). The mucosa was revascularized, as demonstrated on histology (Figure 6.6B and C) and confirmed by blue Microfil injection (Figure 6.6A). Figureure 6.6D shows an enlargement of the integration area with the buccal mucosa and the tracheal mucosa (Figure 6.6D). No differences were seen between the original mucosa of the trachea and the transplanted buccal mucosa (Figure 6.6D).

Group III: porous titanium with vascularized fascia (single stage procedure).

All six rabbits survived until the end of the experiment. The porous titanium was well integrated with the native tracheal cartilage and was fully covered with tracheal mucosa in five of the reconstructed defects. Blue Microfil injection showed revascularisation of the cervical trachea and the reconstructed defects (Figure 6.7A and C).

Histological evaluation of the reconstructed defects showed fully revascularized titanium with pores filled with fibrous tissues and blood vessels (Figure 6.7B and D). Re-epithelialization of the porous titanium from the native trachea resulted in vital looking mucosa and submucosal layer (Figure 6.7E). In one of the reconstructed defects, the titanium was not connected to the cartilage and showed granulation tissue as signs of inflammation around the titanium.

Group IV: porous titanium exclusively.

All six rabbits survived until the end of the experiment. The porous titanium in all reconstructed defects was well integrated with the native tracheal cartilage and was fully covered with tracheal mucosa. There were neither granulation tissue formations nor signs of titanium displacement observed (Figure 6.8A and B).

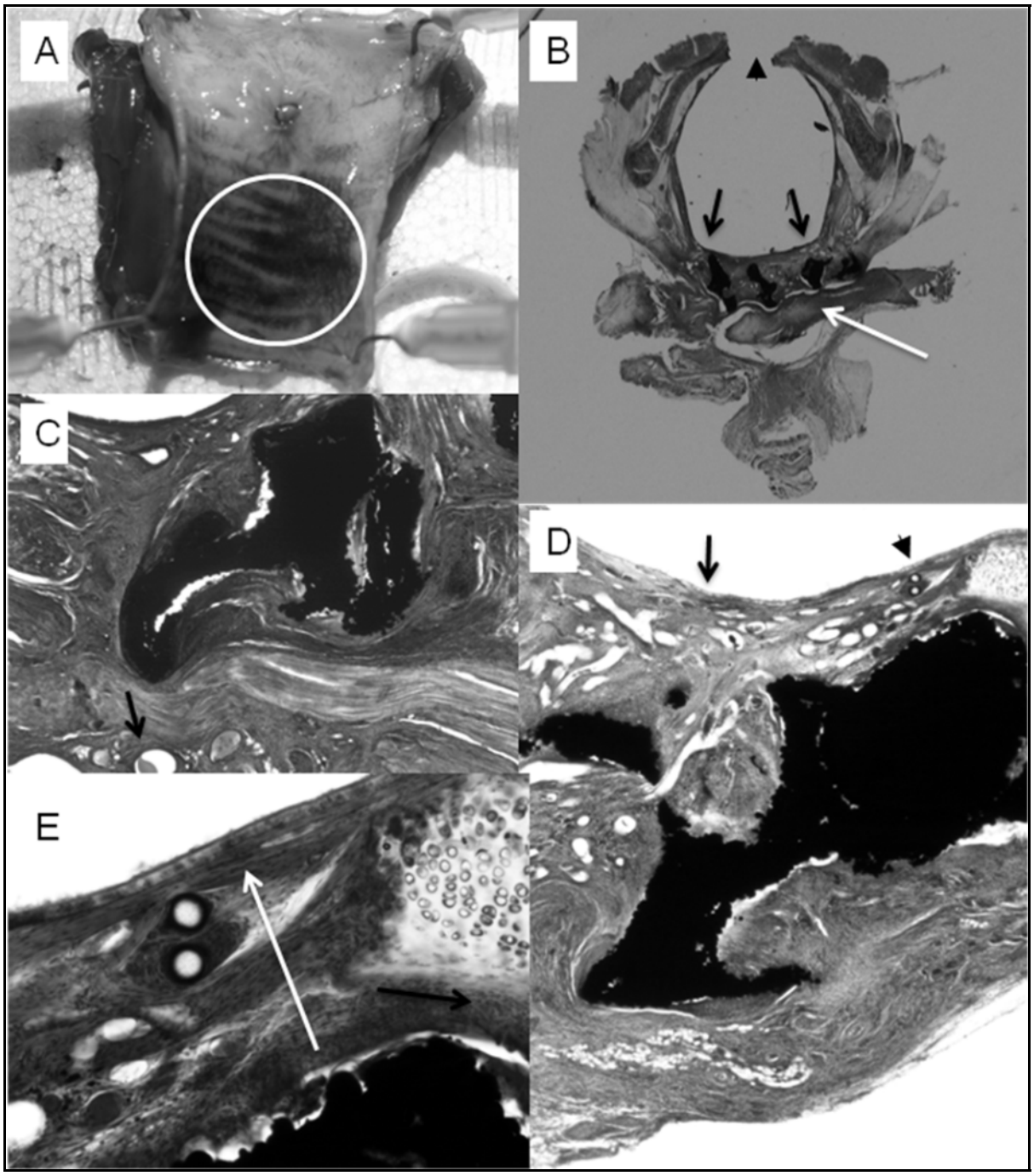

Figure 6.7. Group III: porous titanium with vascularized fascia (single stage procedure). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa. Notice the dark color of the Microfil injection (circled) showing revascularisation of the cervical trachea. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen (black arrows). **C)** Magnification (H&E, original x100) of the pores in the titanium. Notice the presence of the blue Microfil filling the pores of the titanium. The black arrow is pointing to a bloodvessel with blue Microfil. **D)** Magnification (H&E, original x100) of the normal mucosal layer. The picture was taken in the area of the original incision (black arrow). Notice the stitch just submucosally (arrow point). **E)** Magnification (H&E, original x400) of the integration area between the cartilage and titanium. Notice a tight connection is present between the titanium and the cartilage (black arrow). There are clear mucosal and submucosal layers visible, covering the titanium (white arrow) (*see page 125 for color figures*).

Histological evaluation of the reconstructed defects showed fully revascularized titanium with pores filled with fibrous tissues and blood vessels (Figure 6.8C and D). The porous titanium was re-epithelialized from the native trachea with vital looking mucosa and submucosal layer (Figure 6.8D).

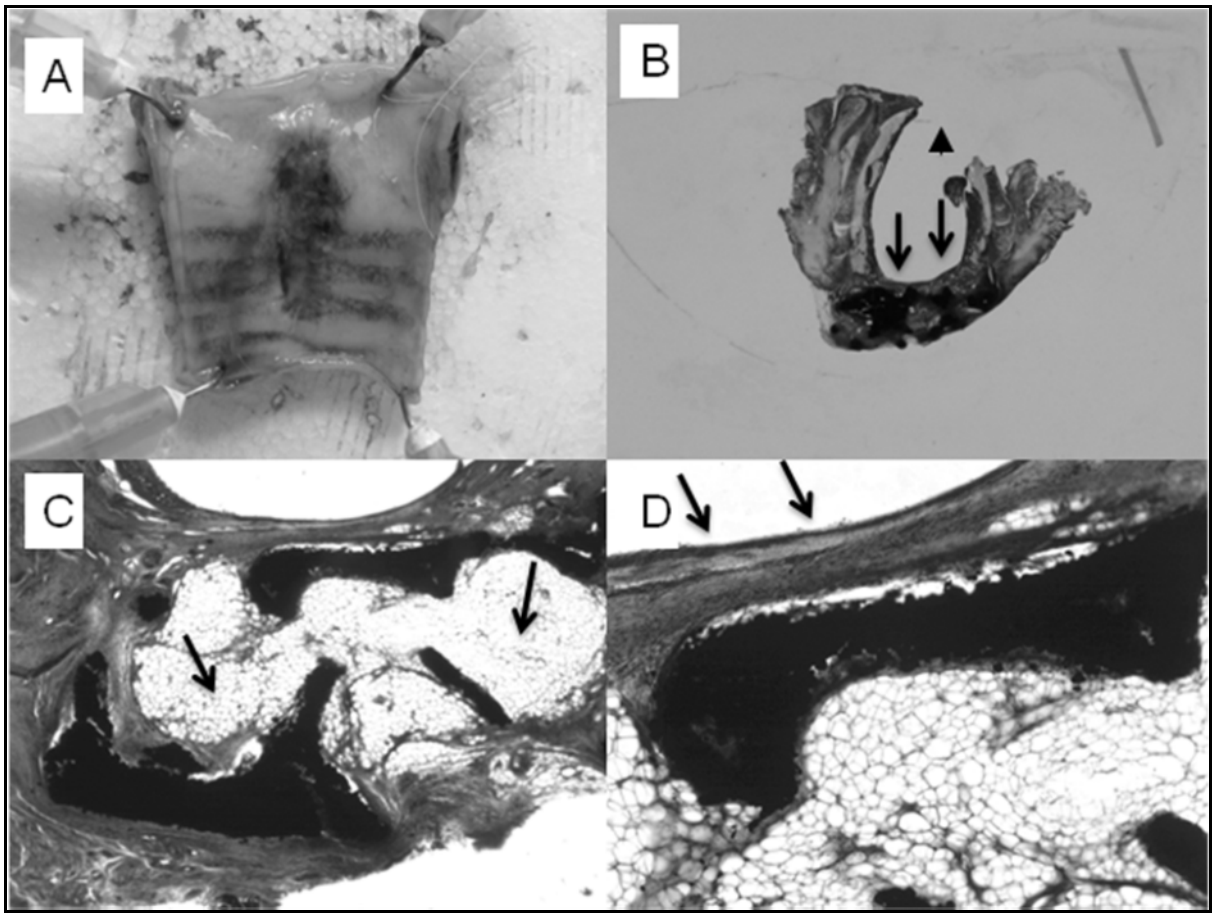

Figure 6.8. Group IV: porous titanium exclusively. A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is visible under the mucosa. The titanium is covered with a thin layer of mucosa. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. Note the integration of the titanium, which provides a concave tracheal lumen. A clear mucosal layer covers the titanium (black arrows). **C)** Magnification (H&E, original x100) of the pores in the titanium. Notice the presence of fibrous tissue filling the pores of the titanium (black arrows). **D)** Magnification (H&E, original x200) showing vital mucosa covering the titanium (black arrows) (*see page 127 for color figures*).

Discussion

The optimal repair tissue for laryngotracheal reconstruction having three basic components, i.e. structural support, mucosal lining, and a vascular supply, does not exist clinically. We need tissue-engineering techniques, prefabrication techniques and the use of biomaterials to replace one or more of these components[14]. Structural

support to keep the airway open can easily be provided by a biomaterial. The biomaterial should allow repair of the mucosal lining, a prerequisite for clinical success. To obtain mucosal regeneration, the biomaterial should allow sufficient vascular supply. Our study demonstrated that laryngotracheal defects can be reconstructed with a porous titanium plate in combination with a vascular carrier and mucosal grafts. Porous titanium was previously used in rats by Schulz et al.[15], who implanted porous titanium in total tracheal defects in 17 rats. Six rats died, five of them because of displacement or sealing of the prosthesis.

Porous titanium combined with a mucosal graft and attached to a pedicled fascia flap can be a good alternative for tracheal reconstruction. This confirmed our previous study[13]. Titanium is sturdy enough to keep the tracheal lumen open and the porous matrix offers the opportunity for blood vessels to grow into the scaffold to revascularize the mucosal graft component. The pedicled fascia flap, on the other hand, provides for definite blood supply for revascularisation of the mucosa through the titanium.

However, is a pedicled fascia flap really necessary to cause re-epithelialization? Or is an additional mucosa (or an epithelial equivalent) graft required to cover the defect? In groups I and II we used autologous mucosa. In group I, the mucosa was left to grow on a piece of titanium over the fascia for 2 weeks. An important drawback of buccal mucosa is the contamination with mouth flora. This leads to the development of granulation tissue as an inflammatory or infectious reaction. In group I, some granulation tissue was found around the stitches. Within group II, some granulations were noticed in two rabbits and two other rabbits succumbed, one due to a titanium implant dislodged outside the tracheal lumen and the other also due to an early dislodged titanium implant. Infection and granulation formation are the limitations, and these problems are frequently cited by other authors. This leads to obstruction of the lumen, air leakage or re-stenosis[16]. A possible solution could be the use of some sort epithelial equivalent, as described by Duff et al.[17], who used cultured abdominal skin punches with collagen type II in a gel for re-epithelialization of a circumferential mucosal defect.

In group III, we only used a pedicled fascia flap and yet the titanium was covered with mucosa in all six rabbits, demonstrating that re-epithelialization occurs from the

wound edges. In group IV we used titanium alone, which was covered with mucosa in all six rabbits. An important point to consider is the size limits of the tracheal defect that requires a pedicled fascia flap to speed up the process of revascularisation. The tracheal defects created in this experiment, which corresponds to about one-third of the total tracheal circumference can be reconstructed with titanium alone. The titanium was covered with mucosa after 6 weeks. For this kind of defects in a rabbit's laryngotracheal complex, a vascular carrier and mucosal graft are not necessary. However, it is probable that bigger defects or circumferential defects require some assistance for their re-epithelialization, as regeneration from the wound edges might be insufficient. Shi et al.[18] created a 45 mm long circumferential tracheal defect in dogs and reconstructed it with different types of prosthesis; they found re-epithelialization only near the anastomotic ends. Delaere et al.[11] found remucosalization of cartilage tubes for segmental tracheal defect only in the area of the anastomosis with a maximum of 3 mm. The question on the size limit of the defect that needs a vascular carrier and/or a mucosal graft is not answered by our study.

Extrapolating these experiments to humans, it might be concluded that in healthy patients and for a small defect, titanium alone might be sufficient. However, patients with fibrosis of the neck area due to prior radiation treatment or previous operations probably need a vascular carrier to ensure the process of re-epithelialization from the wound edges. Larger defects also need a mucosal graft or patches of mucosa that could speed up the process of re-epithelialization. Another possibility is the use of tissue-engineered mucosa that can be added to the porous titanium, as shown by Rakhorst et al.[19].

We did not observe large differences between groups I and II, and III and IV, in terms of mucosal covering of the titanium, although we did observe problems in groups I and II with infection, granulation and titanium displacements.

Another problem we encountered is the way of fixation the titanium. We used Ethilon, four stitches, one at each corner. In most of our rabbits the titanium was well integrated (groups III and IV), although some displacement of titanium into or outside the tracheal lumen was noticed in three rabbits (groups I and II). Fixation of the graft needs further attention, as this was the cause of death of two rabbits in group II.

Using more stitches might prevent collapse but carries the risk of more granulation tissue. Another solution could be the use of an overlay piece of titanium which is fixed outside the trachea and thus cannot collapse inside the lumen.

To our knowledge, this is the first study that made use of porous titanium in a cricotracheal defect in combination with mucosal grafts and a pedicled fascia flap.

Conclusion

Our experiments demonstrate that reconstruction of a rabbit's tracheal defect using composites of porous titanium, mucosal grafts and a fascia flap is feasible. However, it is still a long way before this kind of composite grafts may be used in clinical practice, especially if larger and circular defects have to be reconstructed.

Acknowledgements

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Chapter 7

Summary and general discussion

This thesis deals with some of the problems of laryngotracheal stenosis (LTS). More specifically, it deals with those patients who need laryngotracheal reconstruction after partial resection for benign or malignant tumours, trauma or re-stenosis of the laryngotracheal complex.

Laryngotracheal stenosis

LTS is a growing problem in the field of otorhinolaryngology. Due to improved extensive surgery and better intensive care units, more patients are intubated and require ventilation for extended periods of time. There are many different approaches to treat stenosis, each with its own benefits and disadvantages. The choice for a specific surgical procedure depends on factors such as severity, exact location and overall length of the stenosis. For severe subglottic (grade II, III and IV according to Myer-Cotton scale[1]) and short segment stenosis, the generally accepted surgical procedures of choice are the the cricotracheal split with cartilage interposition grafts or cricotracheal resection (CTR) followed by end-to-end anastomosis.

Single Stage Laryngo Tracheal Reconstruction (SS-LTR). Single Stage laryngotracheal reconstruction as we described in **chapter 2** is a technique in which the stenosis is expanded after anterior and posterior split. In the past, this technique involved placement of intraluminal stents for an extended period of time. At present stenting in SS-LTR procedures would only involve the temporary use of an endotracheal tube[2,3]. CTR on the other hand is a technique in which the stenotic segment is removed and the trachea is anastomosed to the subglottic space. CTR is only possible with some subglottic tissue left to suture the trachea. SS-LTR is in our opinion the treatment of choice for grade II and III stenosis without residual subglottic space. CTR on the other hand is indicated for cases of severe subgottic stenosis with enough subglottic space (grade III and IV). In **chapter 2** we describe 60 patients who underwent a SS-LTR procedure at the Sophia children's hospital in the period January 1994 until December 2006. Fifty-seven patients of the 60 (95%) had a sufficient laryngeal lumen at the time of discharge. This was defined as a situation when the patient could breathe normally without any stridor at rest. Initially, only nine patients (15%) were re-intubated. This decanulation rate of 95% is comparable with

the decanulation rates found in literature after SS-LTR or CTR[4-7]. Cotton et al. reported 86% successful results for SS-LTR in their study in 1992[8] and mentioned a decanulation rate of 90% for all techniques used[9]. Triglia et al.[10] reported 95% decanulation rate in 98 children who were operated on with different techniques including 58 laryngotracheal reconstructions. In our series, patients were extubated as soon as possible with a median duration of intubation of 4 days. Patients extubated after 2 days (two patients) and after 3 days (seven patients) also did well. One patient who extubated himself accidentally 1 day after the operation, was not re-intubated and recovered well. This suggests that it is possible to limit the duration of intubation to less than 4 days. The idea of using a tube as a kind of ''stenting'' does not seem to be necessary with this technique. One can imagine that the longer the tube stays in place, the greater the chance of tissue trauma and thereby impeding the process of remucosalization. SS-LTR should be considered a feasible one-stage and safe operation in selective cases of LTS in children. Our series suggest that intubation time after the operation should be as short as possible.

Cartilage imaging. In **chapter 3** we focused on the cartilage grafts used in laryngotracheal reconstruction. Imaging of cartilage of the larynx with MRI is not yet extensively described in the pediatric literature. MRI of articular cartilage is standard care for diagnosis of articular disorders. MRI of tracheal cartilage grafts in rabbits was previously showed by Delaere et al.[11] in 1999 with high resolution gadolinium enhanced T1-weighted spin echo sequence. We used different MRI techniques with special surface coils to give a high resolution of the cricotracheal complex and thereby making the cartilage more visible. In three patients from our series described in chapter 2, we performed a MRI 3, 6 and 11.5 years respectively after the initial operation. All grafts were visible even after 11.5 years. There was no cartilage-to-cartilage connection but the cartilage grafts were in place and connected to their surrounding with fibrous (scar–like) tissue. No signs of resorption were seen even 11.5 years after implantation. The conclusion seems justified that autologous cartilage with perichondrium will not resorb in time. One possible explanation is that perichondrium provides the cartilage with nutrients. Another possible explanation could be that perichondrium prevents infection of the cartilage and thereby resorption. Zalzal et al.[12] used autologous costal cartilage in rabbits for reconstruction of an anterior split. In the perichondrium group (32 rabbits) no infection was seen. In

the group without perichondrium (15 rabbits) four had infection. Cartilage grafts can be visualized with a MRI-technique using a special surface coil and thin slices based on a gradient echo technique as we described. This can be useful in follow-up of patients in which cartilage grafts are implanted.

Laryngotracheal defects

Surgical repair of the cricotracheal complex in long segment stenosis involving more than half of the entire tracheal length and re-stenosis after an initial resection and end-to-end anastomosis, require some kind of reconstruction. Other patients who need a reconstruction are patients after major trauma, or after resection of a benign or malignant tumour. These kinds of reconstructions remain a therapeutic problem since further resection of the trachea is usually not possible.

Many investigators attempted to create a neo-trachea with cartilage and mucosa. There are some clinical case reports in which a neo-trachea was formed using autologous costal cartilage or seeded chondrocytes in a marlex mesh tube with palatal mucosal graft[13,14]. All patients survived these procedures, but the disadvantage was the need for staged surgery, which is necessary to create the neo-trachea. Herberhold et al. described a series of 112 patients in which preserved allografts from donors were used for tracheal reconstruction in long segment stenosis[15]. However, at present many are reluctant to use homografts because of the Creutzfeld Jacob's disease and other possible infections. It is therefore not considered the first choice of therapy. Recently, Machhianrini et al.[16] described a clinical case where they used a homograft trachea bioengineered with autologous stem cells with success.

Optimal tissue for tracheal reconstruction should contain three different components, i.e. a support tissue to keep the lumen open, a good epithelial lining, preferably consisting of mucosa, and a vascular supply[17]. Ideally, a tissue for laryngotracheal reconstruction would consist of viable cartilage and respiratory mucosal lining similar to that of the native trachea.

Cartilage integration. In **chapter 4** we focused on cartilage-cartilage integration. Cartilage integration is hindered by a lack of matrix producing cells in the interface region. This is mainly caused by chondrocyte death and the inability of chondrocytes to migrate through the extracellular matrix towards the lesion edges, where integrative repair capacity is required. In previous studies, we treated cartilage with highly purified collagenase which provides vital matrix-producing cells near the cutting surface. This was shown to improve integrative cartilage repair[18]. In this chapter, we demonstrated that cartilage integration can be achieved by treating for only one hour using a combination of highly purified collagenase and hyaluronidase. It appeared to be possible to add these two enzymes simultaneously, facilitating clinical application. Furthermore, in an in vivo integration experiment where only the inner core (graft) was enzyme-treated, we showed aside to this increase in vital cell numbers, more frequent integration of the extracellular matrix with matrix fibers crossing over from the enzyme-treated part to the untreated part.

The increase in amount of cells is accompanied by improved integration. In our in vivo integration experiment we saw that 80% of the constructs was integrated (defined by connection between core and outer ring over more than 5% of the interface area), whereas in the control group 46% of the constructs showed integration. We demonstrated that short enzymatic treatment with a combination of hyaluronidase and collagenase increases the amount of vital chondrocytes in wound edges and has a positive effect on integrative cartilage repair. The integration experiment showed (new) fibers crossing over from enzyme treated cartilage to the untreated part. This suggests that enzyme treatment will be favourable for integrative cartilage repair. By reducing the treatment time from 48 hours to 1 hour and treating the graft only, we are a step closer to the possible clinical application of this procedure.

In addition to the cartilage that should form a suitable support tissue, the trachea should also have a good epithelial lining preferably consisting of respiratory mucosa and a vascular supply to support the mucosa. Unfortunately there is no such composite tissue elsewhere in the body that can meet all these requirements. Most of the reconstructive tissues applied clinically lack one or both components leading to the large variation in results. Using tissue-engineering techniques, Delaere et al.[17]

created a prefabricated and prelaminated repair tissue composed of revascularized elastic ear cartilage with buccal mucosal lining and applied it clinically with satisfactory results. A major drawback of this technique is the long waiting period that is necessary to allow revascularisation of the mucosa and remucosalization of the cartilage graft, before the whole regenerated tissue can be finally transferred to the neck area. Cartilage, by nature is avascular and possesses anti-angiogenic properties, making it a less suitable support tissue for mucosal grafts. This was clearly shown by Delaere and Hardillo[19], when they reconstructed circumferential tracheal defects in rabbits with tubed auricular cartilage pre-wrapped in vascularized fascia. Despite the improvement of vascularisation around the tube, healing through remucosalization only occurred around the anastomotic regions leaving the central portions of the grafts bare and partially necrotic.

Biomaterials. To improve reconstruction of the trachea, several experimental approaches have been taken with animal models, such as autologous cartilage with laryngotracheal frameworks from solid titanium, Medpor or hydroxyapatite[20,21], tissue-engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid with or without mucosal lining[22-24] and prefabricated tubes with cartilage strips and a vascularized muscle or fascia[25]. They all had the same problem, that is, the restoration of the mucosal lining through re-epithelialization. This occurred from the wound edges in small defects but for larger defects this process of re-epithelialization proved to be insufficient. The key to the problem might be speeding up the process of revascularisation. This can be achieved with the use of porous materials, in which new vessels can grow through the pores to reach the epithelializing surface to revascularize an implanted mucosal component and thereby ensure its survival. The biomaterial of choice should be strong enough to keep the airway lumen open, and the material should not evoke a foreign body reaction or inflammation. These requirements are met by titanium. Titanium is a material that has been used for years as an implantation material in both orthopedics and maxillofacial reconstructive surgery. Titanium is chemically resistant to the corrosive and oxidative activities of various agents[26]. It has also been shown to be very well tolerated by animal and human tissues making it a suitable material for tracheal reconstruction.

Mucosal survival on porous titanium. In **chapter 5** we investigated the mucosal survival on porous titanium before using it in a rabbit model. This study used porous titanium in combination with mucosal grafts and clearly showed histological evidence of revascularisation of the mucosal graft. After 1 week, new blood vessels were observed growing from the underlying tissue through the titanium scaffold. The presence of the Neuro-Patch eliminated possible revascularisation of the mucosa from the surrounding tissues other than the back muscle. The importance of this revascularisation was also highlighted by the observation that the mucosal grafts in combination with cartilage grafts appeared non vital. We demonstrated that titanium is sturdy enough to keep the lumen open and the porous matrix offers the opportunity for blood vessels to grow into the scaffold. This allows re-epithelialization and survival of a mucosal graft. Titanium has been described to be an ideal biomaterial in a septic environment like the trachea and our rabbit study confirmed this. No inflammatory response or foreign body reactions were observed after 6 weeks of implantation.

Reconstruction with porous titanium. In **chapter 6** we reconstructed a full-thickness laryngotracheal defect using porous titanium in four different ways in an animal model to see if a pedicled fascia flap is really necessary to cause re-epithelialization and to see if an additional mucosa (or an epithelial equivalent) graft is required to cover the defect. In groups I and II we used autologous mucosa. In group I we used a buccal mucosal graft combined with porous titanium implanted on a vascularized fascia flap (first stage) before transposing to the neck area (second stage). In group II porous titanium was implanted on a vascularized fascia flap(first stage) and then transposed to the neck area and combined with a buccal mucosal graft (second stage). An important drawback of buccal mucosa is the contamination with mouth flora. This leads to formation of granulation tissue as an inflammatory reaction. We found granulation tissue around the stitches in both groups I and II. In group II, two rabbits succumbed due to dislodged titanium. Infection and granulation formation are the limitations and problems also frequently cited by other authors. This leads to obstruction of the lumen, air leakage or re-stenosis[27].

In group III and IV we did not use buccal mucosa and we saw no granulation tissue and infections in these groups. In group III, we only used a pedicled fascia flap with titanium in a one-stage operation and yet the titanium was covered with mucosa in all

six rabbits, demonstrating that re-epithelialization occurs successfully from the wound edges. In the last group we only used titanium which was covered with mucosa in all six rabbits at the moment of sacrifice.

These results lead to the question if it is really necessary to use a flap and a mucosa graft. Defects that we created (20 X 5 mm in the anterior cricoid and the first three tracheal rings) which corresponded to about one-third of the total cricotracheal circumference can be reconstructed with single titanium as shown in group IV. The titanium was covered with mucosa after 6 weeks even in the absence of a definite vascular carrier. We should consider which defect's size needs a pedicled fascia flap to speed up the process of revascularisation. Bigger defects or circumferential defects require some assistance for their re-epithelialization, as the regeneration from its wound edges might be insufficient. Shi et al. created a 45 mm long circumferential tracheal defect in dogs and reconstructed it with different types of prosthesis. They found re-epithelialization only near the anastomotic ends[28]. Delaere et al.[19] found remucosalization of cartilage tubes for segmental tracheal defect only on the site of the anastomosis with a maximum of 3 mm. Extrapolating these experiments to humans it might be concluded that in healthy patients and for a small defect (less than one-third in tracheal circumference and involving less than three tracheal rings) single titanium is sufficient. However, for those patients with fibrosis within the neck area due to prior radiation treatment or previous operations, a definite vascular carrier in the form of a pedicled or free vascularized flap might be needed to ensure the process of re-epithelialization from the wound edges. Larger defects (more than one-third in circumference and more than three tracheal rings) probably would also need a mucosal graft or patches of mucosa that could speed up the process of re-epithelialization.

One problem that we encountered, particularly in groups I and II is the presence of infection and granulation tissue formation which could have been brought about by the introduction of buccal mucosa contaminated with mouth flora. This problem can be minimized by using tissue-engineered mucosa in future experiments.

Another problem we encountered is the fixation of the titanium to the native tachea. We used Ethilon sutures, with one stitch at each corner to fix the titanium to the trachea. Using more stitches might prevent collapse but carries the risk of more

granulation tissue. In most of our rabbits the titanium was well integrated (group III and IV) although some displacement of titanium into or outside the tracheal lumen was noticed in three rabbits (group I and II). Fixation of the graft needs further optimization. More stitches could be used. Another possibility is the use of an overlay technique in which the titanium is fixated outside the trachea and therefore cannot collapse inside the lumen.

Conclusion and future perspectives

Surgical intervention in LTS is a challenging aspect in the otorhinolaryngology work field. With the growing incidence of intubated patients, different techniques have been developed and still are being developed. The initial goal of these surgical interventions was decanulation and normal breathing but the goals have expanded to include better voice quality, less invasive techniques and less time in hospital. For SS-LTR we tried to keep the intubation time as short as possible with high success rates.

With modern MRI techniques it is possible to visualize cartilage, even in the young child. This can be useful in the follow up of operated patients with a cartilage graft and gives us a better understanding of the survival of cartilage grafts in time and the integration of the graft with its surrounding. This can be important because a lot of children are operated on a young age and with growth the cricoid and larynx will change with the years.

Cartilage integration can be stimulated by enzymatic treatment. At this time this enzymatic treatment is only used in experimental settings. More studies need to be undertaken to bring back the treatment period to an acceptable time for clinical use. One could think that if this enzyme treatment could be used in laryngotracheal reconstruction, for instance as an enzymatic tissue glue, a better connection is formed between the trachea and the cartilage graft.

For the larger cricotracheal defects that have to be reconstructed, three components are necessary as we mentioned; a support tissue to keep the lumen open, a good epithelial lining and a vascular supply. We think that porous titanium is a promising biomaterial which is sturdy enough and allows revascularisation of mucosa through the pores. A fascia or muscle flap provides the necessary vascularisation of the

mucosal grafts. The problem however of fixation of the titanium safely and permanently on the cartilage of the laryngotracheal structure of the host needs further study.

The problem with infection when using buccal mucosal grafts could be solved by adding antibiotics or by using cultured mucosa harvested in more sterile conditions as mucosal lining. This has been already studied in different ways. Duff et al.[29] used cultured abdominal skin punches with collagen type II in a gel for re-epithelialization of a circumferential created mucosal defect in dogs. They saw severe stenosis (>95%) in the control group and mild stenosis (<20%) for the treated group.

In this thesis we combined in vitro, in vivo and clinical experiments in LTS and reconstruction. For stenosis in children good long-term results of SS-LTR have been demonstrated, making it an excellent technique for most of the patients. With the cartilage studies we performed we have made a step forward in optimal cartilage integration. The imaging of cartilage grafts with MRI will allow us to better evaluate our operation techniques and the survival of cartilage grafts in time. For patients with large defects we are positive about the use of porous titanium which can be combined with mucosal grafts and vascular carries.

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Chapter 8

Nederlandse samenvatting

Dit proefschrift handelt over de problematiek van vernauwingen van het strottenhoofd en de luchtpijp, beter bekend als laryngotracheale stenoses. Meer in het bijzonder deden wij onderzoek naar patiënten die een laryngotracheale reconstructie moesten ondergaan na bijvoorbeeld partiele resectie voor een goed of kwaadaardige tumor, trauma of re-stenose van het laryngotracheale complex.

Laryngotracheale stenose

Stenose van het laryngotracheale complex is een toenemend probleem in de keel-, neus- en oorheelkunde. Door verbeterde operatietechnieken en betere intensive care units, worden meer patiënten geïntubeerd en beademend. Een mogelijke complicatie hierbij is vorming van een stenose. Er bestaan verschillende manieren om zo'n stenose te behandelen, elk met eigen voor- en nadelen. De keuze voor een specifieke chirurgische ingreep hangt af van een aantal factoren te weten: graad van de stenose, exacte locatie en lengte van de stenose. Voor uitgebreide stenose van het gebied direct onder het strottenhoofd, de subglottische stenose (graad II, III en IV volgens de Myer-Cotton schaal) en voor een korte segment stenose is de algemeen geaccepteerde operatietechniek het klieven van het cricotracheale segment, gevolgd door interpostitie met kraakbeen, dan wel cricotracheale resectie en end-to-end anastomose

In **hoofdstuk 2** beschrijven we het laryngotracheale herstel, in het Engels de Single Stage Laryngo Tracheal Reconstruction (SS-LTR), bij kinderen. SS-LTR is een techniek waarbij de stenose wordt gespleten aan de voor- en achterkant waarna de beide helften uit elkaar worden geschoven. Vroeger werd er hierna een stent geplaatst voor langere perioden. Tegenwoordig wordt bij een SS-LTR geen gebruik meer gemaakt van een stent. Het enige ''stenten'' is teruggebracht tot het gebruik van een endolaryngeale tube voor een duur die in dagen kan worden geteld. Onze studie liet zien dat deze duur van de intubatie kan worden teruggebracht tot slechts een paar dagen.

In **hoofdstuk 3** hebben we het lot onderzocht van de kraakbeenstukjes (grafts) die gebruikt zijn bij SS-LTR. Verschillende MRI technieken met speciale coils geven een hoge resolutie van het cricotracheale complex waarmee het kraakbeen beter zichtbaar kan worden gemaakt. Bij drie patiënten uit onze serie beschreven in hoofdstuk 2, werd een MRI gedaan 3, 6 and 11,5 jaar na de initiële operatie. Alle grafts waren goed zichtbaar, zelfs na 11,5 jaar. Er was geen integratie van de beide ingebrachte kraakbeenstukken met het oorspronkelijke kraakbeenskelet maar de kraakbeen grafts waren met hun omgeving vergroeid doormiddel van littekenweefsel (fibreus weefsel). Er werden geen tekenen van resorptie gezien. Concluderend resorbeert autoloog kraakbeen met perichondrium niet met de tijd en kan het zichtbaar gemaakt worden met een speciale MRI techniek. Dit kan bruikbaar zijn in de follow-up van patiënten na gebruik van kraakbeen grafts in laryngotracheale reconstructie.

Laryngotracheale defecten

Chirurgisch herstel is noodzakelijk van een verwijderd cricotracheaal complex dat een lengte heeft van meer dan de helft van die van de trachea, en restenose na eerdere end-to-end anastomose. Andere patiënten die een reconstructie nodig hebben zijn patiënten na een groot trauma of na een resectie van een goed- of kwaadaardige tumor. Deze reconstructies blijven een probleem omdat er niet genoeg weefsel meer over is om te gebruiken voor herstel van het verwijderde gedeelte.

Er is al veel onderzoek gedaan naar reconstructie methoden voor de trachea en larynx. Optimaal weefsel zou uit drie verschillende componenten moeten bestaan, te weten: een ondersteunend weefsel om het lumen open te houden, een goede slijmvlies bekleding aan de zijde van het lumen van de luchtweg en goed doorbloed weefsel dat het geheel voldoende kan voeden. Bij voorkeur zou men moeten kunnen beschikken over kraakbeen-met-luchtwegslijmvlies voor dit soort reconstructies.

In **hoofdstuk 4** onderzochten we de kraakbeen-kraakbeen integratie. Kraakbeenintegratie wordt gehinderd door een gebrek aan matrix producerende cellen in het gebied van de contactvlakken (interface). Dit is hoofdzakelijk het gevolg

van chondrocyten necrose en de onmogelijkheid van chondrocyten om door de extracellulaire matrix heen te migreren naar de interface regio waar zij nodig zijn voor het herstel. In dit hoofdstuk toonden we aan dat kraakbeenintegratie verbetert als het kraakbeen gedurende 1 uur behandeld wordt met een combinatie van de enzymen collagenase en hyaluronidase. In een in vivo integratie experiment zagen we meer vitale chondrocyten, betere integratie en extracellulaire matrixvezels die van het ene stuk kraakbeen naar het andere overgingen. Een goede kraakbeenintegratie is van belang voor de mechanische integriteit van de structuur en voor de groei van de structuur als het een kind betreft.

De luchtpijp heeft ook een goede binnenbekleding nodig. Met gebruik van tissue-engineering technieken is in het verleden een geprefabriceerde samenstelling van gerevasculariseerde stukjes oorkraakbeen en wangslijmvlies met een fascie-lap gemaakt om een defect in de luchtweg te reconstrueren. Het grote nadeel van deze techniek is de lange tijd die het slijmvlies nodig heeft om te revasculariseren. Kraakbeen bevat van nature geen bloedvaten en daardoor zullen er geen bloedvaten door het kraakbeen heen kunnen groeien naar het slijmvlies.

Met het gebruik van poreus materiaal is het wel mogelijk om bloedvaten vanuit de diepere lagen naar het slijmvlies te brengen voor de bloedvatvoorziening (vascularisatie). Dat biomateriaal moet stevig genoeg zijn om de luchtweg open te houden en moet door de omgeving goed verdragen worden zonder dat het een immuunreactie of een infectie opwekt. Deze eigenschappen heeft poreus titanium allemaal. Titanium is een materiaal dat al jaren voor implantatie wordt gebruikt in de orthopedie en kaakchirurgie. Titanium is chemisch resistent tegen corrosieve oxidatie van verschillende stoffen. Het wordt ook zeer goed getolereerd in zowel humaan als dierlijk weefsel waardoor het een geschikt materiaal is voor tracheale reconstructie.

In **hoofdstuk 5** onderzochten we de overleving van slijmvlies op poreus titanium. In een athymische muis werd aan de ene kant op de rugspieren een stukje titanium met wangslijmvlies geplaatst en aan de andere kant een stukje oorkraakbeen met slijmvlies. Al na een week, werden er nieuwe bloedvaten gezien komende van de onderlaag en groeiend door het titanium richting het slijmvlies. Bovendien werd er op het titanium vitaal slijmvlies waargenomen. Op het kraakbeen was het slijmvlies

necrotisch. We toonden in een levend konijn aan dat titanium stevig genoeg is om de luchtweg open te houden en dat er nieuwe bloedvaten door het titanium heen groeien, waardoor het slijmvlies vanuit de wondranden beter en sneller over het titanium kan regenereren. Er werden geen ontsteking of afweerreacties gezien.

In **hoofdstuk 6** reconstrueerden we full-thickness laryngotracheale defecten in konijnen met een combinatie van poreus titanium, slijmvlies en een gesteelde fascie-lap. Het doel was te kijken of een fascie-lap en slijmvlies transplantaat eigenlijk wel nodig zijn voor een defect en of titanium alleen ook kan volstaan. In groep I gebruikten we een wangslijmvliestransplantaat gecombineerd met poreus titanium geïmplanteerd op een gevasculariseerde fascie-lap (eerste operatie) voordat we het geheel naar de hals transporteerden (tweede operatie) voor het herstel. In groep II werd voor de reconstructie gebruik gemaakt van poreus titanium dat geïmplanteerd werd op een gevascullariseerde fascie-lap (eerste operatie) en daarna verplaatst naar de hals, samen met een wangslijmvliestranplantaat (tweede operatie).

In deze twee groepen zagen we veel granulatie weefsel als uiting van ontsteking, waarschijnlijk vanuit en door het wangslijmvlies. In groep III en IV hebben we geen slijmvlies gebruikt. In groep III, hebben we poreus titanium gebruikt met een facie-lap in een enkele operatie. In groep IV hebben we alleen poreus titanium gebruikt. In deze groepen hadden we geen problemen met ontsteking en granulatievorming.
Deze resultaten laten zien dat voor de defecten die wij gecreëerd hebben (20 X 5 mm in het anterieure cricoid en de eerste drie trachea ringen) uitsluitend titanium voldoende is zoals we zagen in groep IV. Waarschijnlijk is voor grotere defecten of voor circulaire defecten titanium alleen niet voldoende. De vraag is vanaf welke grootte van het defect een slijmvliestransplantaat en/of extra bloedvoorziening wel nodig is

Een ander probleem dat nog opgelost moet worden is de fixatie van het titanium. Wij fixeerden met vier hechtingen maar in twee konijnen in groep II was het titanium naar binnen losgeschoten. Meer hechtingen of toepassing van een soort ‘’overlay’’ techniek van het titanium kunnen dit mogelijk voorkomen.

Conclusies

Chirurgische interventie in laryngotracheale stenose vormt een uitdaging binnen de keel-, neus- en oorheelkunde. Er zijn verschillende herstelmogelijkheden waarbij de SS-LTR een techniek is met goede resultaten.

Met moderne MRI technieken is het mogelijk om kraakbeen zichtbaar te maken, ook in een jonger kind. Dit kan nuttig zijn voor evaluatie van laryngotracheale reconstructies waarbij kraakbeengrafts werden gebruikt. Het kraakbeen resorbeert niet, zelfs niet op lange termijn.

Kraakbeen-kraakbeen integratie kan gestimuleerd worden door enzymbehandeling. Meer onderzoek moet gedaan worden om de klinische toepasbaarheid te testen. Hierbij kan gedacht worden aan ontwikkeling van een soort enzymlijm die bijvoorbeeld bij de SS-LTR toegepast kan worden voor een betere kraakbeenintegratie waardoor de mechanische integriteit en de vorm van het kraakbeenskelet beter is.

Voor grote cricotracheale defecten zijn drie componenten nodig voor een geslaagde reconstructie. We denken dat poreus titanium in combinatie met slijmvlies en een vorm van bloedvoorziening een goed alternatief zou kunnen zijn voor de grotere defecten. Problemen die nog overwonnen moeten worden zijn de kans op infectie bij slijmvlies grafts en de fixatie van het titanium in zijn omgeving.

In dit proefschift hebben we in vitro en in vivo laboratoriumstudies gecombineerd met klinische studies met als doel vernauwingen en defecten van de luchtweg en het strottenhoofd door middel van reconstructieve technieken te behandelen. In de toekomst zullen echter nog verdere verbeteringen moeten worden aangebracht.

Dankwoord

Ik wil graag een aantal mensen bedanken die het tot stand komen van dit proefschrift en promotie hebben mogelijk gemaakt.

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Janella. Mijn vrouw sinds 11 december 2009. Lieve Janella. Totdat ik in het Rode Kruis ziekenhuis voor mijn perifere stage begon had ik niet het idee dat ik iemand zou tegen komen waar ik zo onvoorwaardelijk voor zou gaan. Sindsdien is mijn leven weer compleet. Je hebt me gesteunt tijdens de opleiding en laatste loodjes van dit proefschrift. Je geeft me rust, gezelligheid, lol en veiligheid in het leven. Danki dushi. Mi stima bo hopi!

Curriculum Vitae

Luuk Janssen werd op 15 november 1972 geboren te Utrecht. In 1991 behaalde hij het eindexamen VWO aan de Katholieke Scholengemeenschap de Breul te Zeist. In dat zelfde jaar begon hij aan zijn studie geneeskunde aan de Rijksuniversiteit Utrecht. Zijn wetenschappelijk stage werd uitgevoerd bij de afdeling Anatomie waar hij ook een aantal studentassistent schappen deed onder andere het practicum Hoofd-Hals Anatomie. Na het artsexamen in 2000 heeft hij 14 maanden als agnio KNO/Chirurgie in het Antoni van Leeuwenhoek ziekenhuis te Amsterdam gewerkt. Daarna is hij begonnen als agnio KNO op de afdeling KNO/Hoofd-Hals Chirurgie in de Daniel den Hoed kliniek te Rotterdam. Aansluitend heeft hij anderhalf jaar onderzoek gedaan voor dit proefschrift en is 1 juli 2004 gestart met de opleiding tot KNO-arts aan de Erasmus Universiteit te Rotterdam. De opleiding is voltooid op 1 juli 2008 en sindsdien is hij bezig met de KNO vervolgopleiding oncologie en chirurgie (KNOVOO) aan het Universitair Medisch Centrum te Utrecht. In het kader van dit fellow ship is hij 6 maanden in het Antoni van Leeuwenhoek ziekenhuis geweest en aansluitend 4 maanden in het Rajavithi ziekenhuis in Bangkok, Thailand. Vanaf 1 juli zal hij zijn carrière voortzetten als KNO-arts Oncologisch Hoofd-Halschirurg in het Universitair Medisch Centrum Utrecht. De auteur is getrouwd met Janella en zij wonen in Utrecht.

Color figures

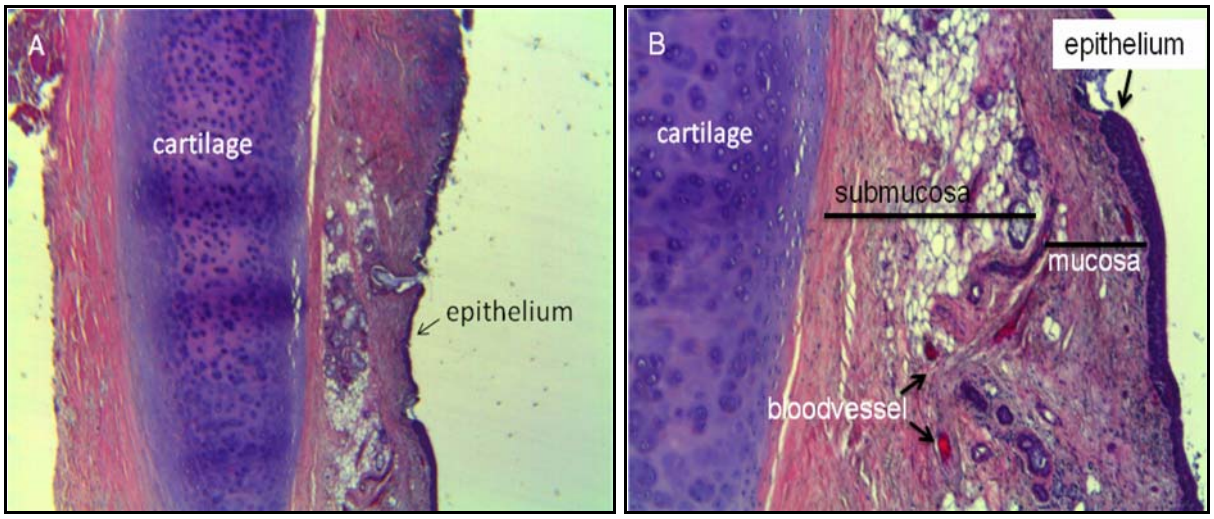

Figure 1.2. Cross-section of human trachea (H & E). **A)** Magnification, (original x10) with submucosa and respiratory epithelium. **B)** Magnification, (original x40) shows the different layers of the epithelium and cartilage.

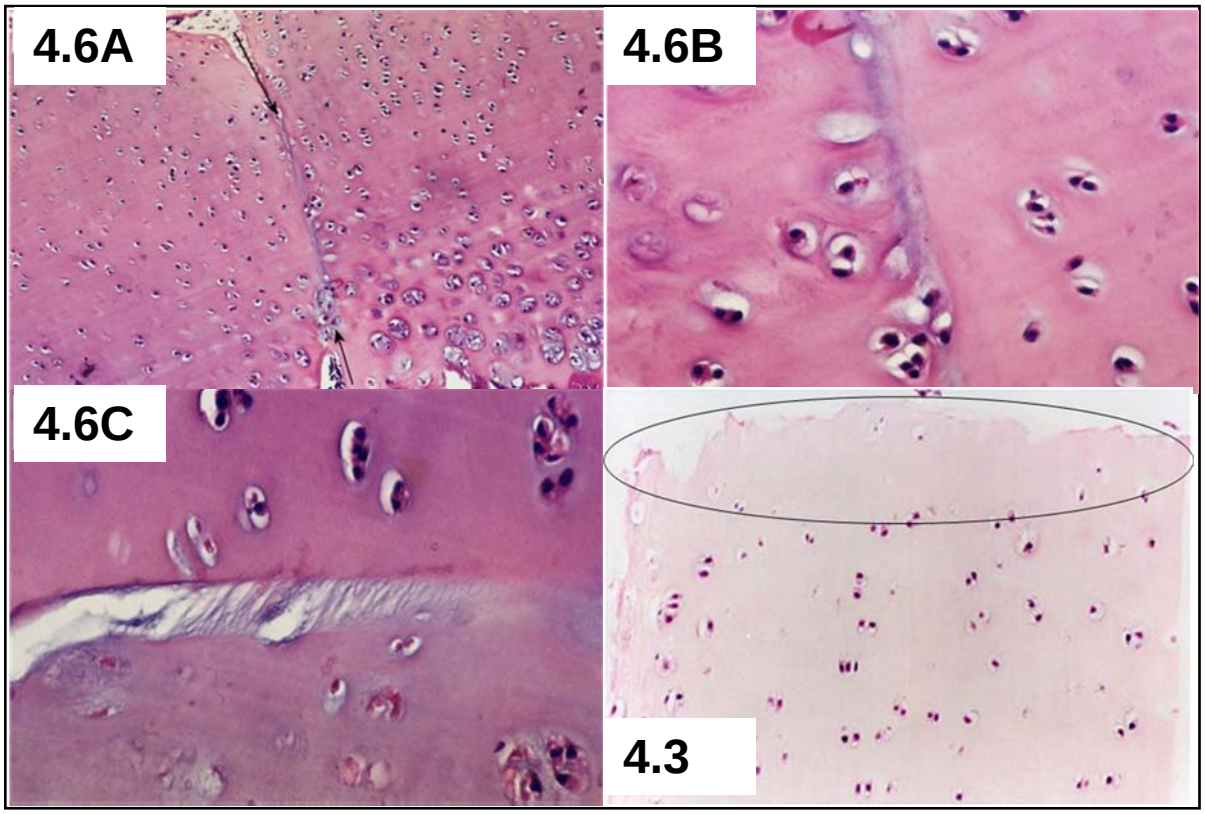

Figure 4.6. Zone of the enzyme treated cartilage in integration experiment. **A)** Chondrocyte density at the interface of treated inner core (right) and untreated outer ring (left; H&E, original x100). Integration area (arrows) shows almost 100% integration. **B, C)** Magnification of the integration area (H&E, original x400). **B)** More vital chondrocytes are visible in the enzyme-treated inner core (left) in comparison with untreated outer ring of the cartilage (right). **C)** collagen fibers are crossing over from the treated part (bottom) to untreated part (top).
Figure 4.3. Chondrocyte death in the wound edges (H&E, original x100). Fourteen days after wounding acellular band is visible at wound edge (circled). Left is the superficial layer and right is deep layer.

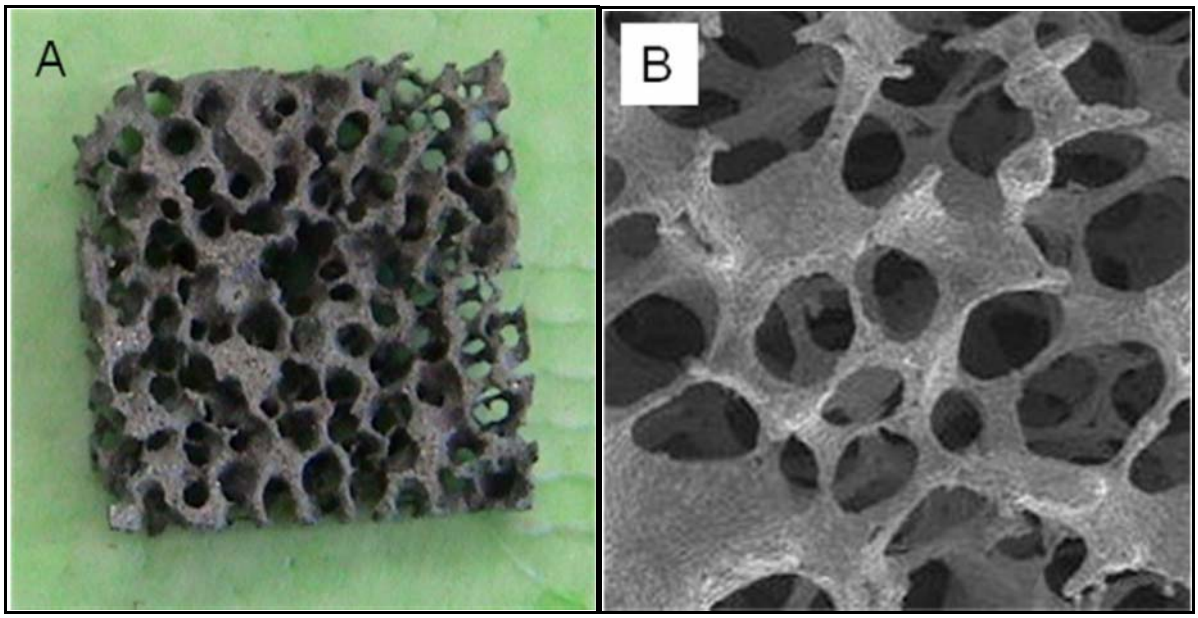

Figure 5.1. A) Titanium plate of 10 X 10 X 2 mm. with pores ranging from 400 to 700 µm and a porosity of 90%. **B)** Magnification of Figure A (original x10).

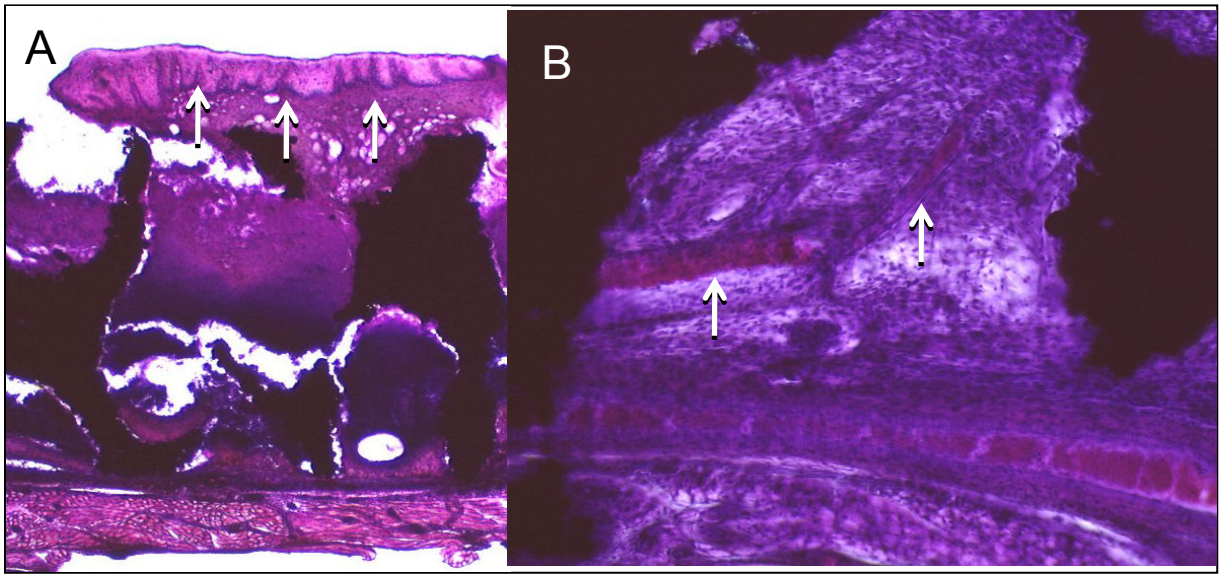

Figure 5.3. Histological section of human buccal mucosa on top of titanium after 2 weeks of implantation. **A)** Human buccal mucosa (H&E original x2.5). The black material is titanium. The mucosa is attached to the titanium. The pores of the titanium are filled with fibrous tissue and blood vessels. The white arrows point to the basal layer of the mucosa. **B)** Magnification (H&E original x10) showing the titanium filled with fibrous tissue and blood vessels (white arrows).

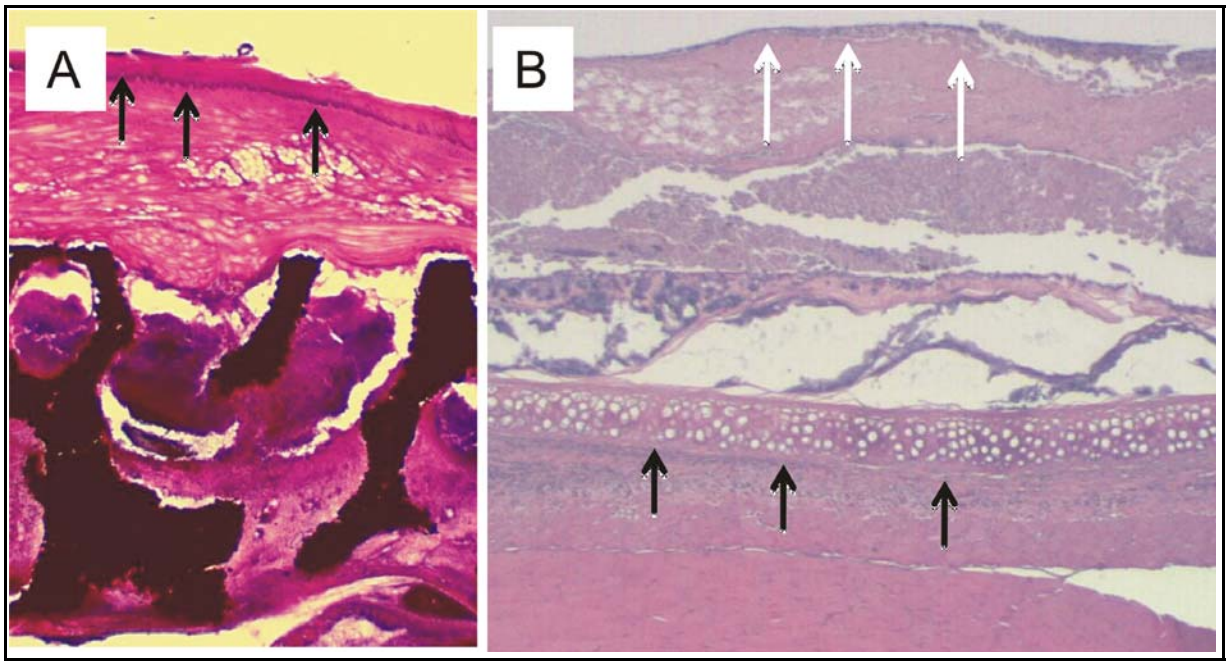

Figure 5.4. Rabbit buccal mucosa. **A)** Rabbit buccal mucosa on top of titanium (H&E magnification original x25). Note the intact basal layer as indicated by the black arrows. **B)** Rabbit buccal mucosa on top of ear cartilage (H&E magnification original x25). In the middle of the picture the cartilage is visible with its chondrocytes (black arrows). There is no connection between the mucosa and the cartilage. The basal layer of the mucosa is not visible (white arrows).

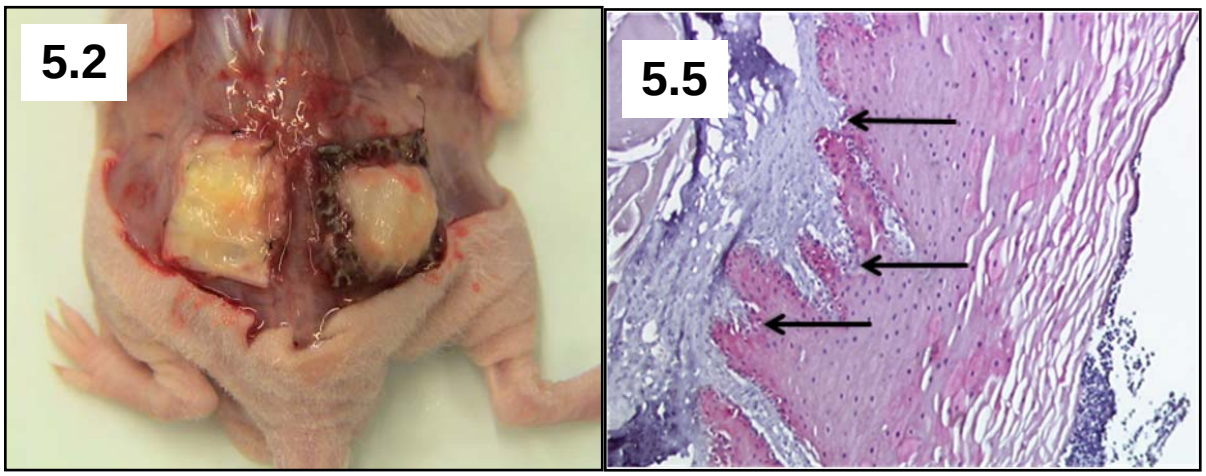

Figure 5.2. The back of an athymic mouse with on the left a cartilage piece with rabbit's buccal mucosa. On the right, a titanium plate with rabbit's buccal mucosa. This is post termination after 2 weeks of implantation.

Figure 5.5. Cytokeratin staining (CK13) on rabbits buccal mucosa after 2 weeks of implantation with a clear basal membrane (black arrows) and suprabasal keratinocytes of vital mucosa (original magnification x100, visualization with New Fuchsin, counterstain with Gill's haematoxylin).

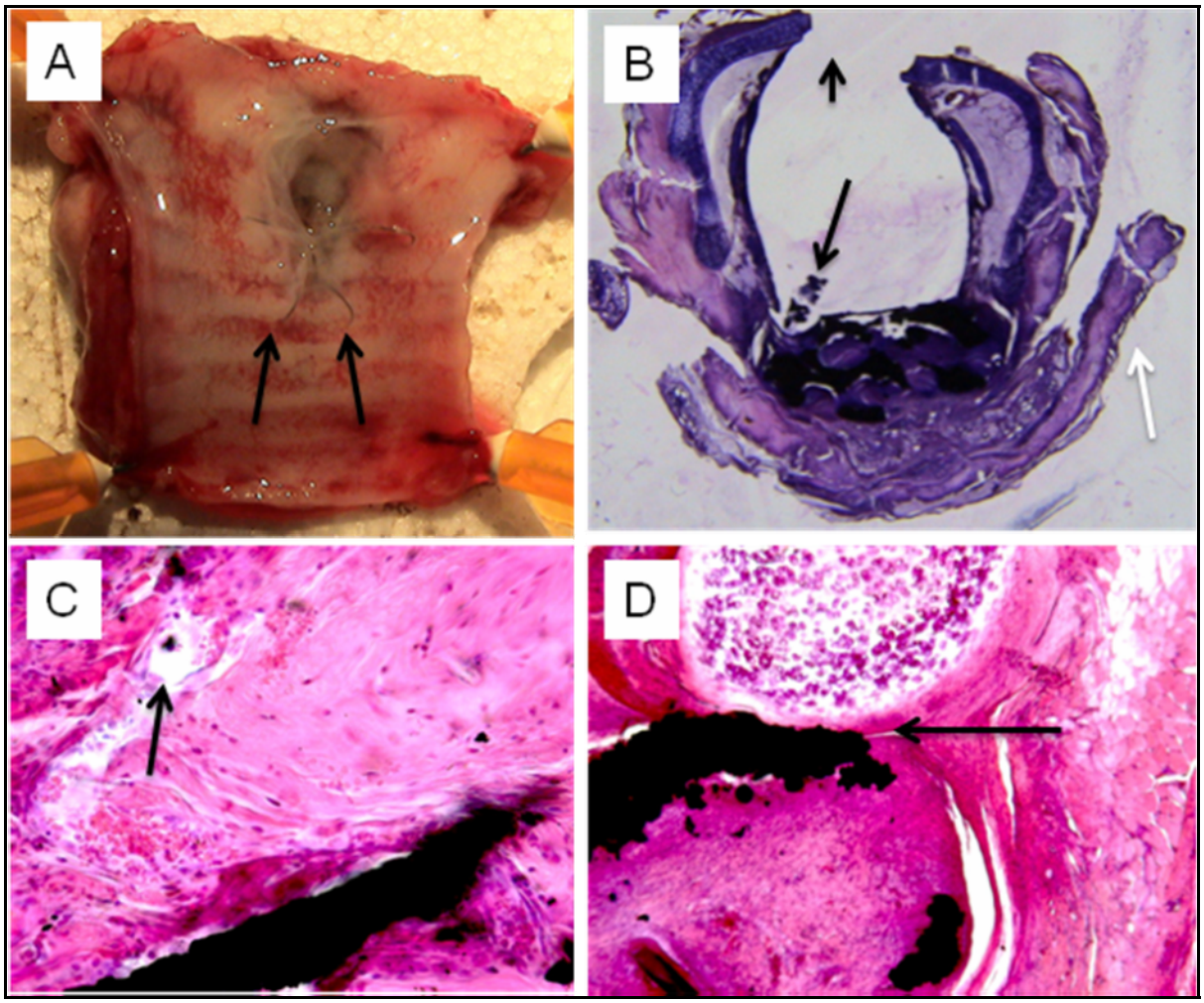

Figure 5.6. Cricotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. **A)** The titanium is visible under the mucosa with stitches (black arrows). The titanium is covered with a thin layer of mucosa. **B)** Histological cross-section through the trachea (H&E). The short black arrow indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. A thin layer of mucosa is covering the titanium. Some granulation tissue is present on the left side of the titanium just inside the tracheal lumen (long black arrow). **C)** The pores in the titanium (H&E, original magnification x200). Notice the presence of fibrous tissue filling the pores of the titanium. The black arrow is pointing at a bloodvessel. **D)** The integration area between the cartilage and titanium (H&E original magnification (x100). Notice a tight connection is present between the titanium and the cartilage (black arrow).

Figure 6.3. Schematic drawing of a rabbit with the titanium plate placed on the left lateral thoracic fascia flap.

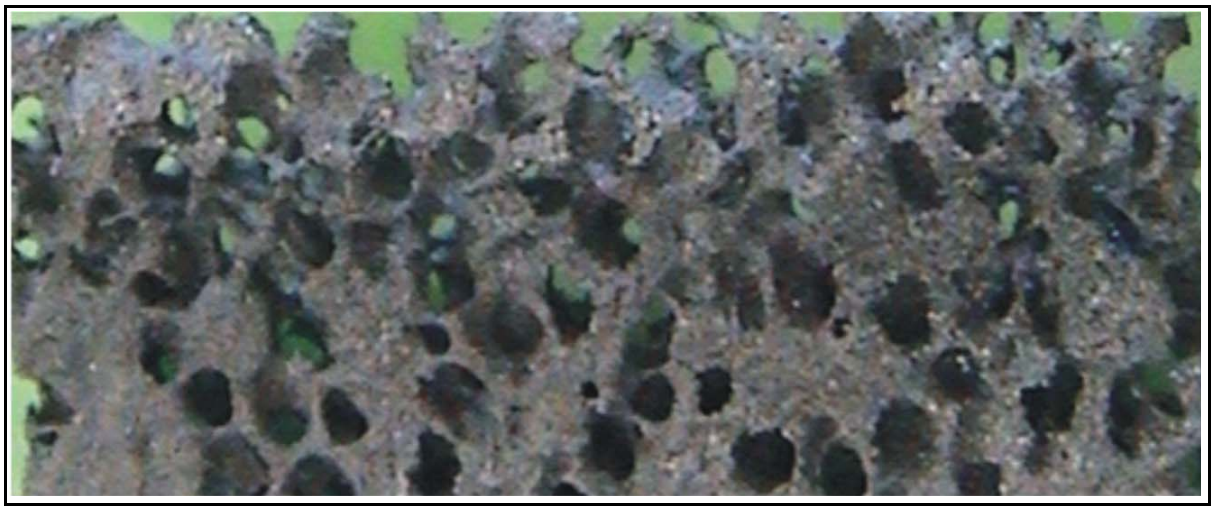

Figure 6.1. Curved titanium plate of 20 X 5 mm and a thickness of 2 mm, with pores in the range 400-700 ụm and a porosity of 90%.

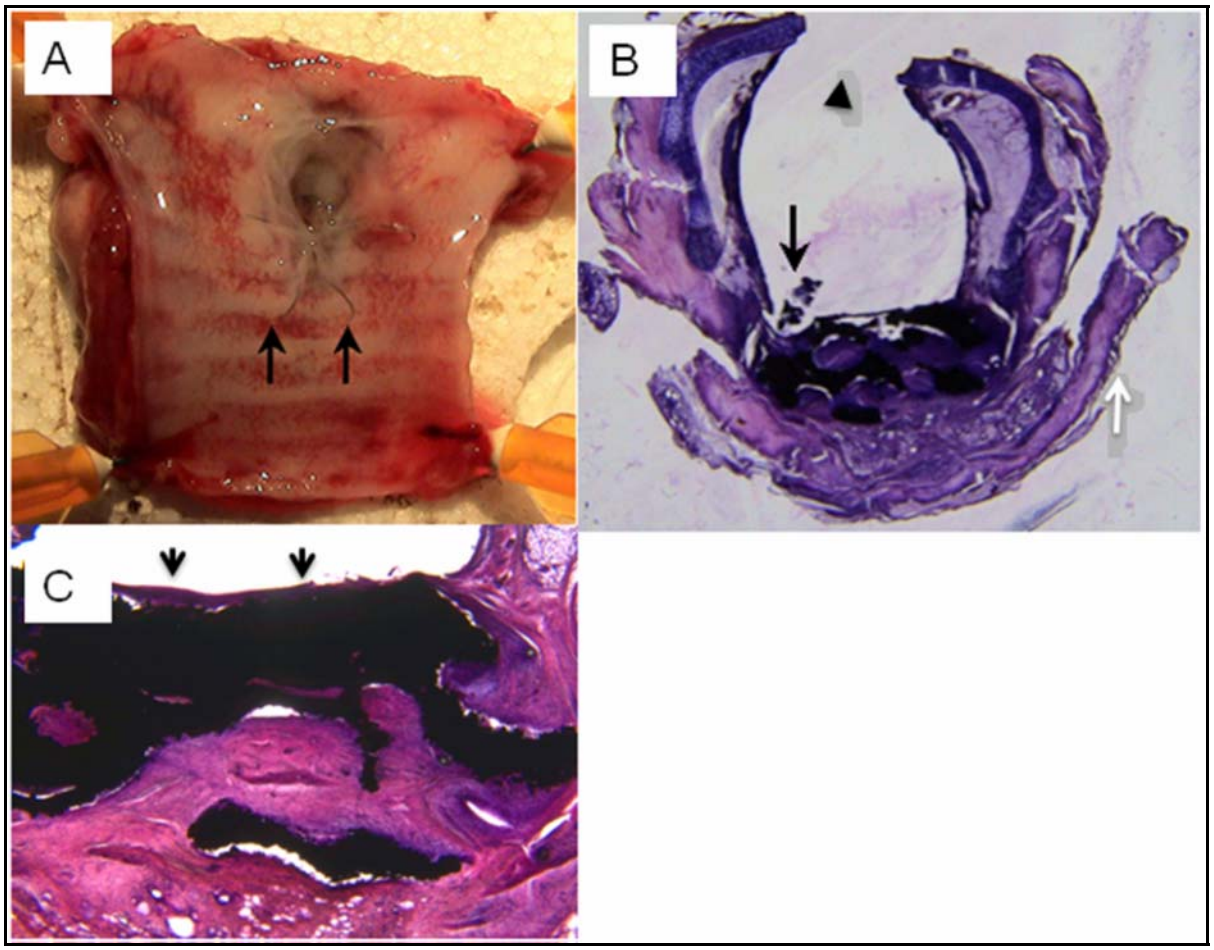

Figure 6.5. Group I: porous titanium initially implanted on a vascularized fascia and combined with buccal mucosal graft (first stage) before transposing to the neck area (second stage). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa (black arrows). **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. The black arrow is pointing at the integration area, with some granulation tissue on the mucosa visible. **C)** Magnification (H&E, original x100,) of the integration area between the native tracheal mucosa and the mucosal graft. There is a thin mucosal layer visible covering the titanium (black arrows).

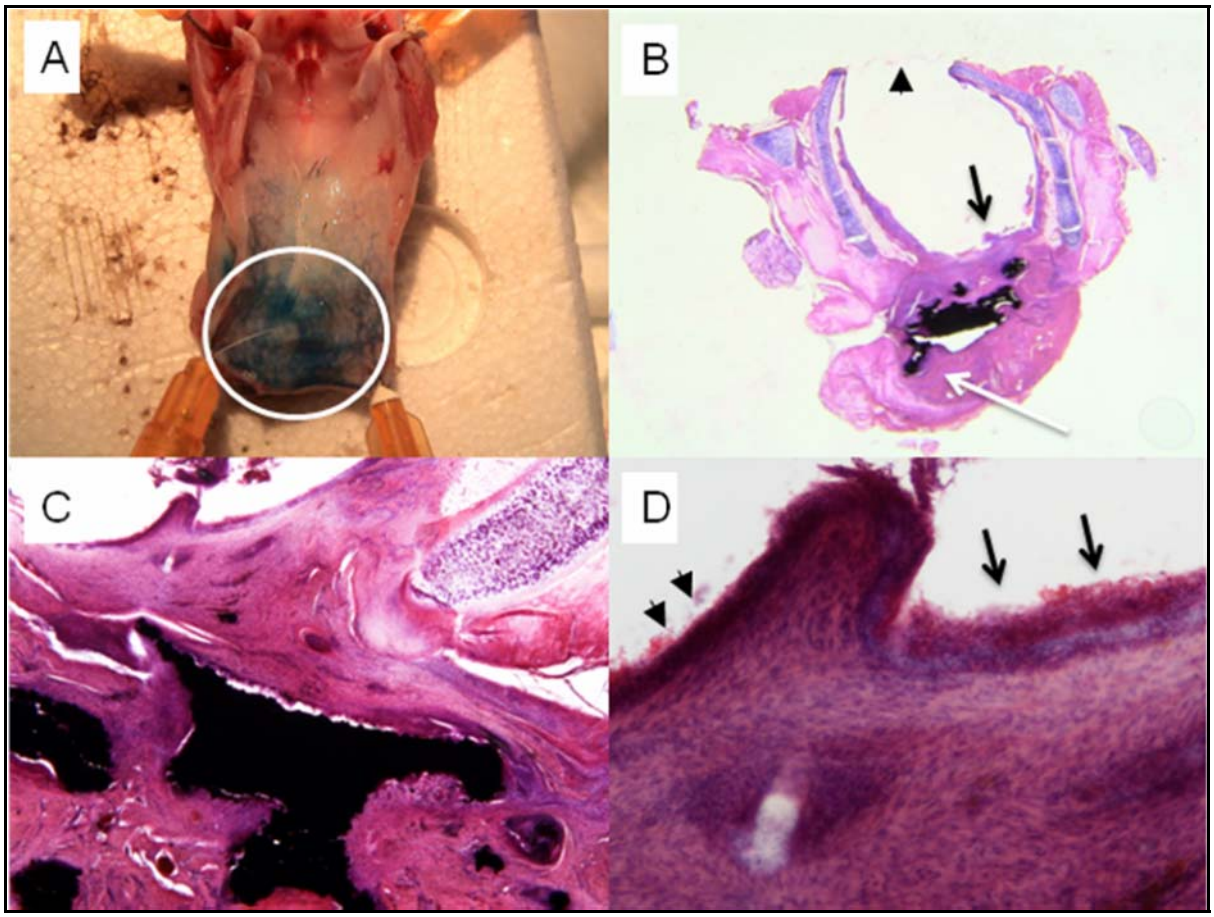

Figure 6.6. Group II: porous titanium implanted on a vascularized fascia (first stage) and combined with a buccal mucosal graft (second stage). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa. Notice the dark color of the Microfil injection (circled) showing revascularisation of the cervical trachea. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen. The black arrow is pointing at the integration area, with some granulation visible on top of the mucosa. **C)** Magnification (H&E, original x100) of the integration area between the native tracheal mucosa and the mucosal graft. There are clear mucosal and submucosal layers visible, covering the titanium. **D)** Magnification (H&E, original x400) of the integration area between the native tracheal mucosa (black arrows) and the buccal mucosal graft (arrow points).

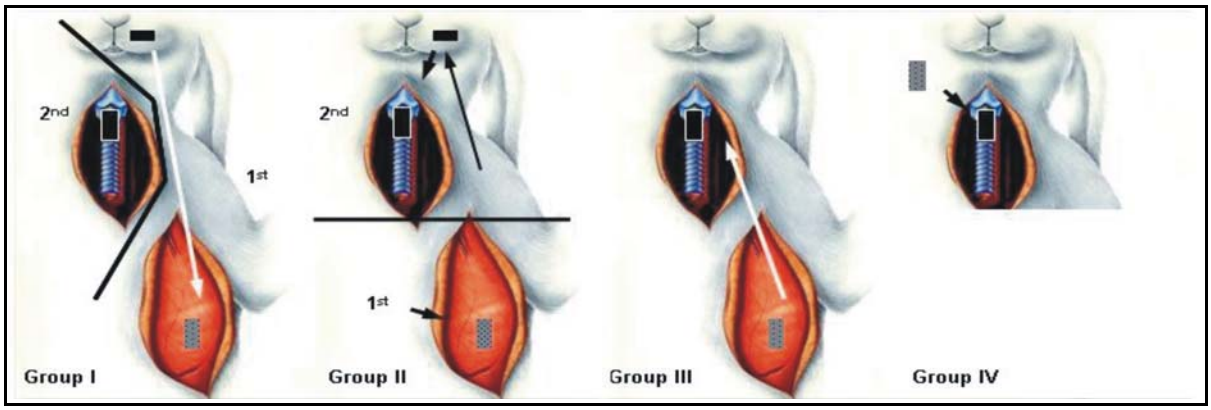

Figure 6.4. Schematic drawing of the four different groups. Groups I and II are operated on using a two-stage procedure; groups III and IV are operated on using a one-stage procedure.

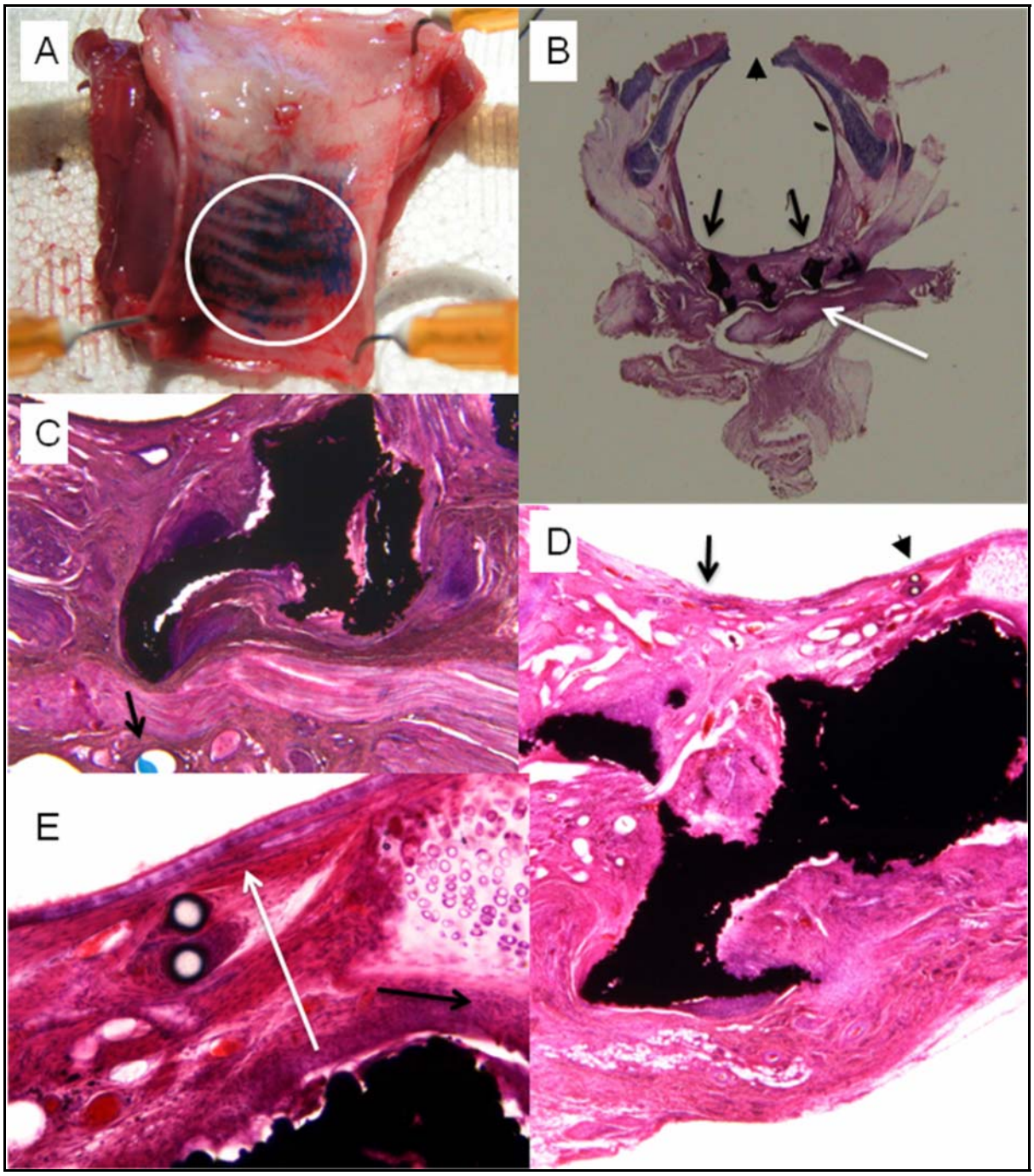

Figure 6.7. Group III: porous titanium with vascularized fascia (single stage procedure). A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is covered with a thin layer of mucosa. Notice the dark color of the Microfil injection (circled) showing revascularisation of the cervical trachea. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. The white arrow indicates the pedicled fascia. Note the integration of the titanium, which provides a concave tracheal lumen (black arrows). **C)** Magnification (H&E, original x100) of the pores in the titanium. Notice the presence of the blue Microfil filling the pores of the titanium. The black arrow is pointing to a bloodvessel with blue Microfil. **D)** Magnification (H&E, original x100) of the normal mucosal layer. The picture was taken in the area of the original incision (black arrow). Notice the stitch just submucosally (arrow point). **E)** Magnification (H&E, original x400) of the integration area between the cartilage and titanium. Notice a tight connection is present between the titanium and the cartilage (black arrow). There are clear mucosal and submucosal layers visible, covering the titanium (white arrow).

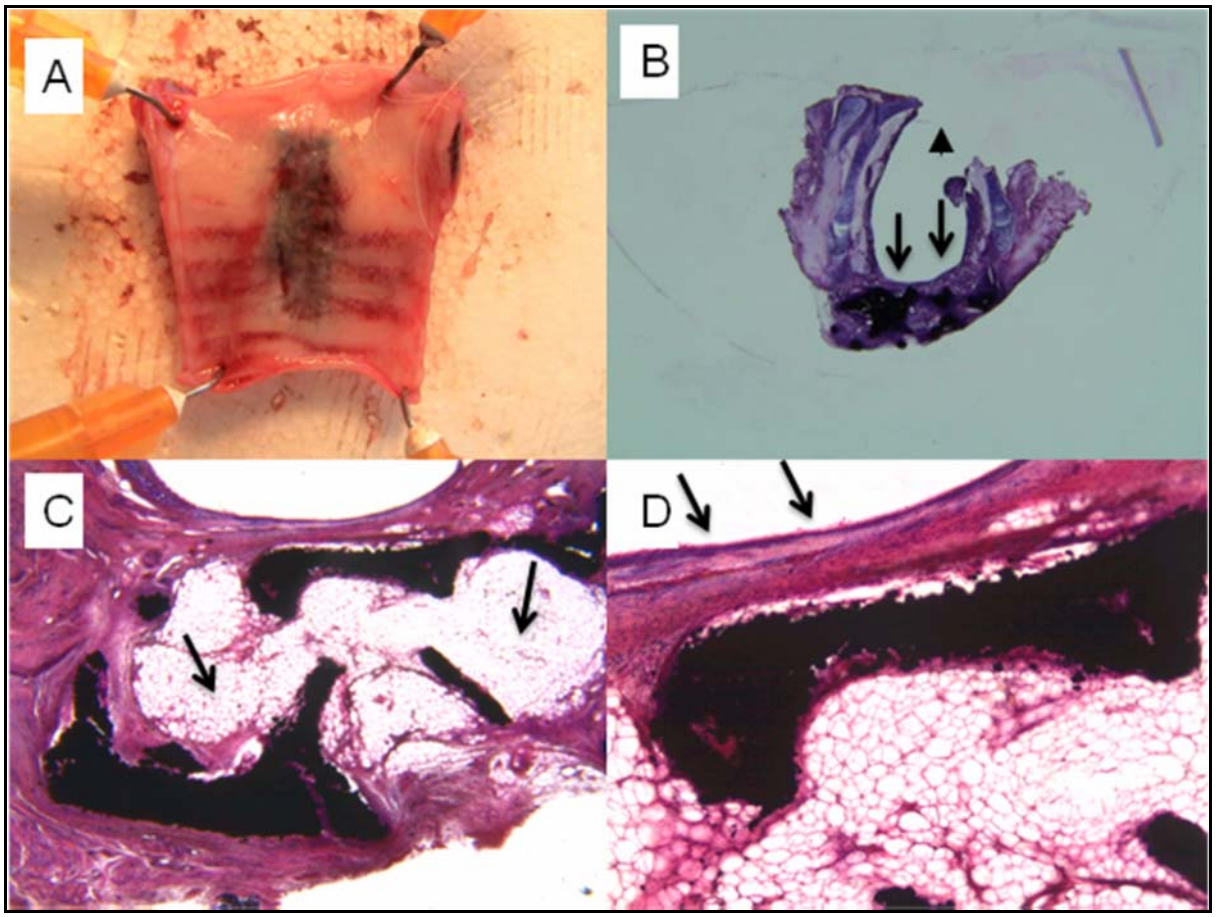

Figure 6.8. Group IV: porous titanium exclusively. A) Laryngotracheal complex of the rabbit after 6 weeks, cut open in the posterior plane. The titanium is visible under the mucosa. The titanium is covered with a thin layer of mucosa. **B)** Histological cross-section through the trachea (H&E). The arrow point indicates the opening in the posterior plane. Note the integration of the titanium, which provides a concave tracheal lumen. A clear mucosal layer covers the titanium (black arrows). **C)** Magnification (H&E, original x100) of the pores in the titanium. Notice the presence of fibrous tissue filling the pores of the titanium (black arrows). **D)** Magnification (H&E, original x200) showing vital mucosa covering the titanium (black arrows).

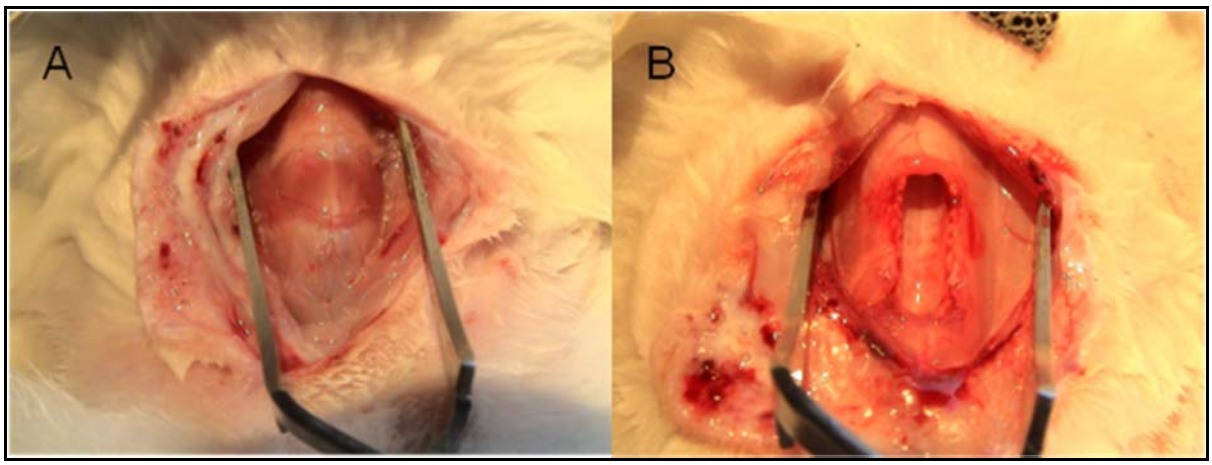

Figure 6.2. Cervical area of the rabbit exposed **(A)** before and **(B)** after creation of the full-thickness defect (through cartilage and mucosa) measuring 20 X 5 mm in the anterior cricoid and the first three tracheal rings. The defect is one-third of the total circumference. The head is downwards in this image.