ACQUIRED SUBGLOTTIC STENOSIS

an experimental study

Cover: Demineralized Bovine Bone Matrix, 3 weeks after implantation in a vascularized perichondrial pocket.

ACQUIRED SUBGLOTTIC STENOSIS
an experimental study

DE VERWORVEN SUBGLOTTISCHE STENOSE
een experimentele studie

PROEFSCHRIFT
ter verkrijging van de graad van Doctor
aan de Erasmus Universiteit Rotterdam
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This study is part of the project Airway Stenosis .
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Ter nagedachtenis aan mijn moeder
Aan mijn vader, Sharon en familie
Wendy en Gabriëlle

CONTENTS

CHAPTER 1

INTRODUCTION

Subglottic (endolaryngeal) injury can cause a subglottic stenosis. Chronic subglottic stenosis is defined as a partial narrowing (to complete obliteration) of the airway bounded by the inferior margin of the cricoid at the caudal side and cranially by the insertion of the fibres of the conus elasticus into the true vocal cords. Subglottic stenosis may be congenital or acquired. A congenital subglottic stenosis is the remnant of an incomplete recanalization of the laryngeal lumen after completion of normal epithelial fusion at the end of the third month of gestation [1]. Mostly, a stenosis at the level of the subglottis is acquired and considered to be the consequence and thus the complication of an extra- or endolaryngeal injury to the larynx. An external trauma causes fractures of the cartilaginous skeleton with lacerations of the soft tissues. An acquired subglottic stenosis following prolonged endotracheal intubation develops in 0.9 - 8.5 % of prematurely born neonates who need artificial respiration [2,3], is often more severe and now forms the largest proportion of cases in infants and children [4,5].

The most vulnerable segment of the airway appears to be the cricoid ring, being the narrowest part, with the overlying epithelium en subepithelial tissues (fig. 1). A subglottic stenosis involves the loss of soft tissue lining, including the elastic mantle, glands and vessels, and erosion of the inner perichondrial layer and subperichondrial cartilage of, especially, the cricoid cartilage and sometimes also one or more of the tracheal rings. Probably an acquired subglottic stenosis develops along the following sequence of events: Initially trauma is induced by insertion of the endotracheal tube. This causes microscopic mucosal hemorrhage and edema. Continued pressure ulcerations occur, most commonly in the posterolateral parts of the cricoid ring. Occasionally, ulcerations are circumferential. With increasing trauma or duration of intubation the ulcers enlarge and extend deeper into the surrounding tissues. Granulation tissue is formed in the bases of these lesions; ciliary stasis and local infection increase the inflammatory response, resulting in more granulation tissue formation [6,7]. In this tissue collagen fibres are deposited which create a fibrous thickening.

In children as well as in adults these complications can occur, but children are most frequently affected. In all cases the morbidity of a subglottic stenosis is a considerable burden for the patient. Moreover, the treatment often concerns long-term, mostly multi-sessional procedures which recognize successes but also failures. Multi-disciplinary medical centers of high quality are preferable.

Although acquired laryngotracheal stenosis now is a well-documented complication of

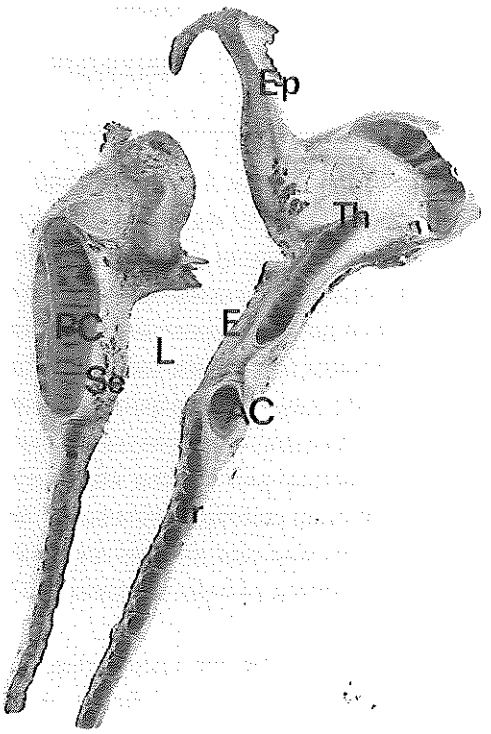

Figure 1
Longitudinal section through the normal larynx of a 3- year-old boy. Subglottic area is indicated. Note that the narrowest part of the airway lumen is at the level of the cricoid ring.
Ep = epiglottis
Th = thyroid cartilage
AC = anterior part of cricoid ring
PC = posterior part of cricoid ring (lamina)
Tr = tracheal rings
L = airway lumen
E = epithelium
Se = subepithelial layer.

endotracheal intubation, recommendations for methods of treatment vary considerably. More conservative therapies like dilation, stenting and intubation are used next to surgical methods which often comprise cricoid splits, with or without the interposition of cartilage grafts. The latter procedure is the treatment of choice at present.
Initially the different surgical approaches for treatment were often based upon empirical experience and for many years have lacked sufficient insight into the histopathological processes and wound reactions during the period of wound healing. Especially the growing larynx (in young and particularly premature children) has to be managed very carefully. The factor of laryngeal growth in children merits prominent consideration during the course of clinical investigations and planning of the different surgical procedures.
Holinger et al. who in 1976 made a clinical histopathological classification, founded on the post-mortem investigation of human specimens, distinguished a diversity in hard and soft stenoses and suggested that the therapy should be adjusted to the type of stenosis [8].

The question arises why so many different types of stenosis are encountered. Moreover, it should be noted that the factors involved, do not have to be similar at an early age -during growth- as later in life.

In a series of earlier published experiments Adriaansen et al tried to find an answer to this question [9,10,11,12,13]. This study was restricted to the growing larynx in young rabbits. Some of the most important conclusions are:

1. The larynx is constructed according to the concept of two concentric tubes, the cartilaginous skeleton and the soft tissue layer. Remarkably, this has never been taken into account in clinical publications on subglottic stenosis.
2. The complete cartilaginous cricoid ring is not indispensable for the patency of the subglottic lumen, provided that the inner tube of elastic fibres (tunica elastica) and other parts of the cartilaginous skeleton are intact. The elastic fibres cannot regenerate.
3. Endolaryngeal trauma can cause different types of stenosis corresponding to Holinger's observations and classification.
4. The type of stenosis is dependant upon the depth (= degree) of the injury:
   a. trauma limited to the soft tissue lining will result in a moderate stenosis encompassing bands of more circularly oriented fibrous tissue.
   b. trauma including the inner perichondrium and subperichondrial cartilage will lead to a severe stenosis due to dense fibrotic scar tissue, ectopic cartilage and a collapse (= deformation) of the cricoid ring.
5. A different type of deformation is observed when the cricoid ring is interrupted. It was defined as "stretching" of the fragments due to a certain turgor present in the cartilage, first described by Gillies [14] and later by Fry [15] as interlocked stresses.

The investigations of the present study have been started on the basis of the above-mentioned findings.

First, the hypothesis that the release of interlocked stresses through splitting of the cricoid ring or trauma of the inner perichondrium of the cricoid cartilage plays a role in the deformation of the cartilage, was tested (chapter 2, 3 and 4).

Secondly, the long-term effects of a regularly clinically utilized combined anterior and posterior cricoid split on form and size of the cricoid fragments were assessed (chapter 5).

The influence of age and ageing in relation to the reaction of the subglottic structures on specific and similar injuries are subject of chapter 4 and 6.

The possibility to reconstruct lost parts of the cricoid cartilage in the rabbit has already been studied by Adriaansen et al [16], Lapidot [17] and Zalzal [18]. Whereas the latter used costal (Zalzal) and thyroid (Lapidot) cartilage in adult animals, Adriaansen investigated the use of hydroxylapatite grafts to reconstruct the anterior cricoid arch. He noticed that hydroxylapatite has an excellent biocompatibility and is readily incorporated in the surrounding tissues. It also became clear however that this biomaterial is not suitable for application during growth. In the course of time the cricoid reconstructed with a hydroxylapatite graft will develop a

subglottic stenosis because the growth of the posterior part of the cricoid ring is not able to compensate for the static anterior graft.

In chapter 7 and 8 the usefulness of another biomaterial i.e. demineralized bovine bone matrix (DBBM) which is transformed into a growing cartilaginous graft and applied for reconstruction of the cricoid ring in a one- or two-stage procedure, is demonstrated.
The closure of a complete defect in the anterior subglottic laryngeal wall, using the same porous alloplast DBBM is described in chapter 9.
Finally, chapter 10 summarizes the conclusions and statements of this thesis.

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CHAPTER 2

TRAUMA OF THE CRICOID AND INTERLOCKED STRESS

ABSTRACT

In young rabbits the effects were studied of an anterior midline and a bilateral cricoid split with and without traumatization of the perichondrium and subperichondrial cartilage on the inner side of the ring. Various interventions produced specific patterns of distortions. It is concluded that release of interlocked stresses in the cartilage is of paramount importance for the development of deformities. A specific feature of the circular cartilaginous structure seems to be that the tensile forces on the outer side of the ring exceed those on the inner side.

INTRODUCTION

Specifically aligned tensile stresses have been demonstrated in human costal and nasal septal cartilage [1-3]. It was suggested that the outer layers of the tissue are maintained in tension so that the intact cartilage has a balanced system of forces, called "Interlocked stresses",the resultant of which is zero [4]. Carving the surface on one side will partially release the interlocked stress of the opposite side. This causes the cartilage to curl towards the intact side as has been shown in vitro by Fry [5]. Furthermore, he observed that the degree of deformation depends upon the thickness of the cartilage. In general thin cartilage deforms more than thick parts.

In live young rabbits the immediate overlap of the cut edges after dissection of the cartilaginous nasal septum was considered to be another manifestation of interlocked stress [6]. It should be stated that interruption of cartilage and traumatization on one side led to different types of distortion, but both were supposed to originate from the above mentioned system of interlocked stresses.

The question whether interlocked stress can equally be observed in other cartilaginous structures, like the skeletal components of the larynx, seems relevant to the hypothesis of forces in cartilage. The present investigation is concerned with the question whether interlocked stresses are present and how they contribute to deformities of the cricoid ring which develop after various traumata, and is part of a long-term series of experiments on cricoid growth and subglottic stenosis.

METHODS

The basic methodology in this study has previously been described [7,8] and is only summarized below. Forty young (4-week-old) female, New Zealand White rabbits, weighing approximately 450-500 g each were used in 4 groups of 10 animals each. Anaesthesia was induced with Xylazin-hydrochloride 2% [Rompun] (1 ml/kg) and Ketamin 10% (1 ml/ kg)

IM. The cricoid was approached via a midventral incision through the skin and subcutis; the overlying muscles were retracted laterally during surgery.

DESCRIPTION OF THE EXPERIMENTAL SERIES PERFORMED

SERIES A: An anterior median cricoid split through the cricoid without damaging the underlying soft tissues.

SERIES B: A bilateral cricoid split, approximately 3 mm on either side of the midline, without disturbing the soft tissue lining of the subglottic lumen.

SERIES C: Traumatization of the inner surface of the cricoid arch (perichondrium and subperichondrial cartilage) with a burr [9], followed by an anterior median cricoid split.

SERIES D: Traumatization of the inner surface of the cricoid arch (same procedure as series C) combined with a bilateral and anterior median split, dividing the cricoid arch in two fragments.

Muscles, subcutaneous layer and skin were approximated and sutured (catgut) in separate layers. At the adult stage (20 weeks later) the animals were sacrificed. The larynx specimens were excised and processed histologically; 5μ thick transverse sections were prepared and stained with Pas-Alcian blue. The sections were assessed microscopically.

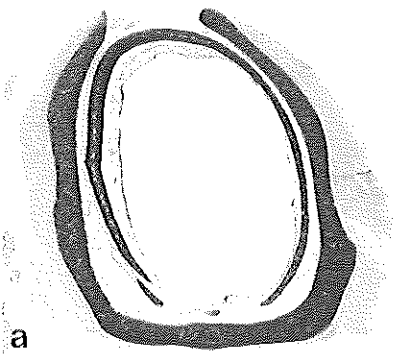
RESULTS

ANTERIOR CRICOID SPLIT

SERIES A Figs. 1a and 2: In all animals, immediately after surgery the cut ends were observed to recede in lateral direction, leaving a gap of approximately 1 mm. This gap remained present up to the adult stage. Reconnection of both stumps was never found. In the adult specimens, the histological aspect of the cartilage appeared to be normal

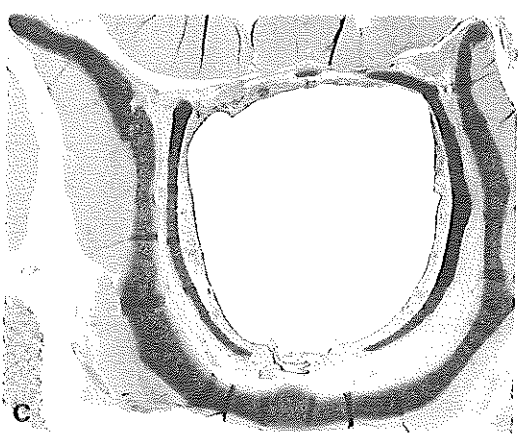
BILATERAL CRICOID SPLIT

SERIES B Figs. 1b and 3: In the adult stage the anterior and posterior fragments of the cricoid ring showed marked changes in shape. In both parts straightening had taken place, leading to distinct flattening of the ventral arch and a U-like configuration of the dorsal fragment with a sideward inclination of the lateral parts. In some cases the anterior segment was positioned in between the stumps of the dorsal part, causing evident rounding of the airway lumen.

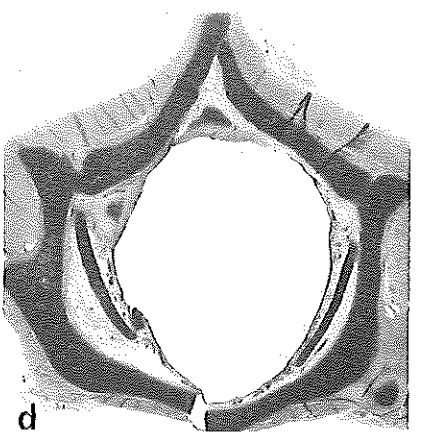


Anterior cricoid split (Series A)

Bilateral cricoid split (Series B)



Traumatization of the inner surface layer of the ventral part and anterior cricoid split (Series C)



Traumatization of the inner surface layer of the ventral part with anterior and bilateral cricoid split (Series D)

Figure 1
Transverse view of the cricoid of a rabbit, 20 weeks after surgery (x 6).

TRAUMATIZATION OF THE INNER SURFACE OF THE CRICOID ARCH AND ANTERIOR MIDLINE SPLIT

SERIES C Figs. 1c and 4: The anterior stumps of the cricoid were separated by a wider gap than in Series A and appeared to curl outwards. Actually those parts of the cricoid, of which the inner surface (perichondrium and subperichondrial cartilage) was injured, had lost their normal curvature and - referring to the lumen - changed from concave to convex. Also, in this series the posterior part of the cricoid ring showed a U-like configuration.

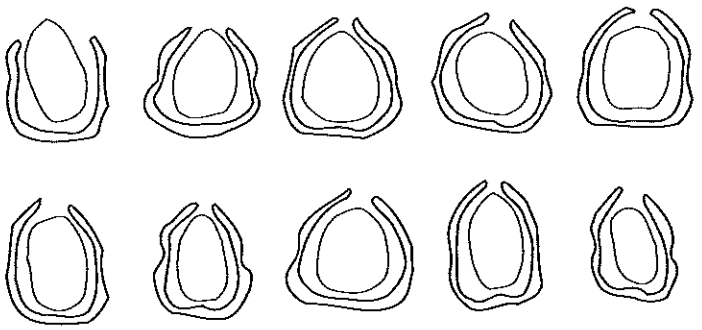


Figure 2
Schematic illustration of the cricoids in Series A. In all specimens a gap is noted of varying diameter.

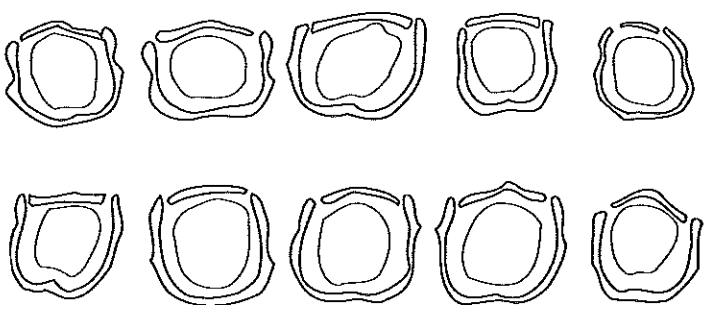


Figure 3
Schematic illustration of the results in Series B. Flattening of the ventral part and U-like configuration of the dorsal part.

TRAUMATIZATION OF THE INNER SURFACE LAYER OF THE CRICOID ARCH, COMBINED WITH A MIDLINE AND BILATERAL SPLIT

SERIES D figs. 1d and 5: Both anterior fragments of the cricoid ring demonstrated a convex form (referring to the lumen), resulting in an inward collapse, leading to a stenosis of the airway. The posterior part of the cricoid ring had developed a U-like configuration.

DISCUSSION

The normal development during the experimental period [9,10] is characterized by an increase in size of the cricoid ring and a change in shape - from almost round to elliptical in the dorso-ventral axis - without significant thickening of the cartilage (fig. 6).

The various types of injury of the cricoid did not cause grossly diminished growth of the cartilage. They resulted in specific patterns of deformities described for the series A,B,C and D. The evolution of these deformities has not yet been studied. They reflect immediate

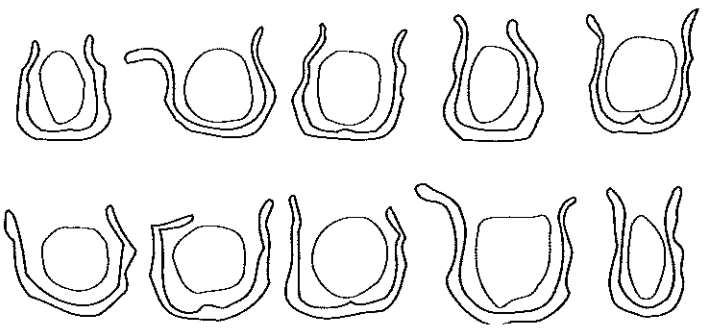


Figure 4

Schematic illustration of the results in Series C. Warping outwards of the ventral cut ends is found in most specimens.

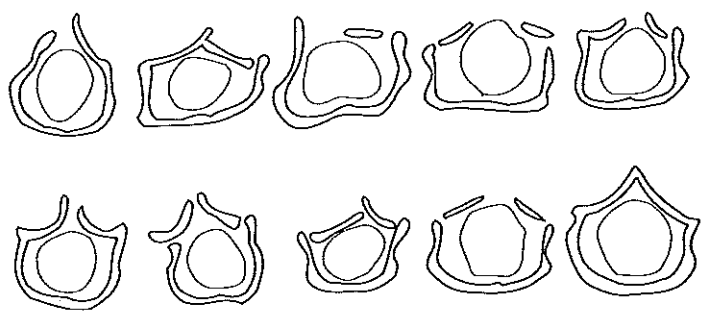


Figure 5

Schematic illustration of the cricoids in Series D. In most cases the fragments of the ventral arch demonstrate distinct curling with the convexity towards the lumen.

reactions to the surgical interventions and further postnatal development. Only in case of the anterior midline split (Series A) the sideward retraction of the cut edges was observed to occur immediately.

Can these deformities be related to the release of interlocked stresses? Fry [5] considered interlocked stress as a "turgor" due to the waterbinding capacities of the protein polysaccharides in the cartilage matrix. This turgor induces tensile forces, especially in the collagen skeleton of the subperichondrial cartilage [2]. Fragments of the cricoid ring (Series C and D) appear to react in just the same way as cylindrical and plate-like cartilaginous structures [1,2] to destruction of the perichondrium and subperichondrial layer on one side: they show curling to the undamaged side. Fragments of a bilaterally split cricoid ring (Series B) show a tendency to straighten. The anterior fragment is in most cases almost straight. The posterior fragment shows a sideward inclination of the lateral parts: widening to a U-like configuration. This "straightening" of both fragments supports the hypothesis that the tensile forces in

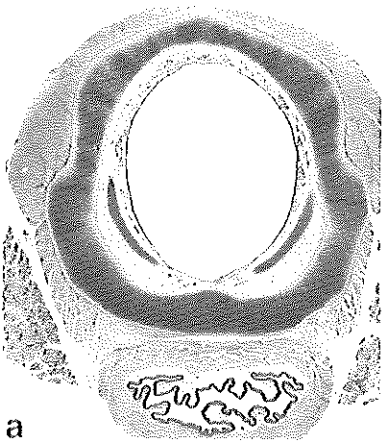


Figure 6a
Transverse section through the normal subglottis in a 4-week-old rabbit with a more or less round shape of the cricoid ring. Dorsolaterally in the subepithelial layer the upper part of the first tracheal ring (x 10).

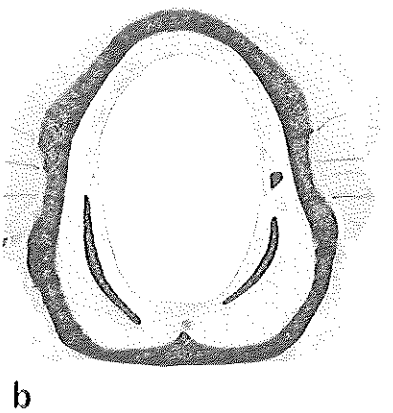


Figure 6b
Transverse section through the cricoid cartilage of a 24-week-old rabbit. The cricoid ring and subglottic airway lumen have increased in size, mainly in sagittal diameter (x 6).

the outer layer of the cricoid ring exceed those on the inner layer. Such a preponderance of tensile forces on the outer side also explains the gap between the cut edges after an anterior midline cricoid split (Series A).
The reactions to various interventions were more obvious in the anterior than in the posterior part of the cricoid ring. This is probably due to differences in thickness and stability of the cartilage [5]. The arch is proportionally thin, whereas the posterior lamina is large, particularly in the cranio-caudal dimension, and therefore more resistant.

The results in Series C and D further demonstrate that the effect of interrupting the cricoid ring, and destruction of the perichondrium and subperichondrial cartilage, on the inner side are cumulative.

CONCLUSIONS

1. Splitting of the growing cricoid and traumatization of the inner side of the ring (perichondrium and subperichondrial cartilage) induce specific deformities.
2. These deformities can be considered to be the result of a release of interlocked stress. The concept of Gibson [2] and Fry [5] is adapted to a circular structure by adding the hypothesis that tensile forces on the outer surface exceed those on the inner side of the ring.

3. Splitting of the cricoid causes widening of the (interrupted) cricoid ring and straightening of the fragments. Destruction of perichondrium and subperichondrial cartilage leads to curling to the undamaged side. Both effects are cumulative.

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CHAPTER 3

THE INFLUENCE OF DIFFERENT TYPES OF SPLITS UPON THE GROWING CRICOID

ABSTRACT

Splitting of the cricoid is a frequently used procedure in the management of a subglottic stenosis. Different types of cricoid splits are introduced for specific forms of pathology. An anterior cricoid split (ACS), bilateral cricoid splits (BLCS) and a combined anterior and posterior cricoid split (APCS) are most approved clinically. Despite the intensive use of cricoid splits, much research has not been dedicated to observations of the morphological changes induced by these procedures. In this study different types of cricoid splits were performed in young rabbits: - a unilateral cricoid split, - a bilateral cricoid split and fragmentation of the anterior cricoid arch into four pieces. A comparative study of the morphological outcome of these procedures was executed.

INTRODUCTION

Splitting of the cricoid in the treatment of a subglottic stenosis in children is common practice nowadays. This form of laryngotracheal surgery was initiated in 1956 by Réthi [1], who introduced his technique of a combined anterior and posterior cricoid split in young patients with remarkable results. It meant the start of a new era with respect to the management of laryngotracheal stenosis. New surgical procedures evolved in which splitting of the cricoid turned out to be a key manoeuvre. In 1972 Fearon and Cotton [2] described a clinical successful technique, after prior experimentation in primates, involving an anterior cricoid split with the interposition of a costal cartilage graft. Two years later, in 1974, Evans and Todd [3] introduced a new form of laryngotracheoplasty, using a castellated incision of the skeleton of the anterior subglottic wall. Splitting of the cricoid is an essential part of this procedure. In 1980 an anterior cricoid split was proposed by Cotton and Seid [4] for neonates who experienced a difficult decannulation. Reports in 1988 [5] and 1991 [6] detailed the evolution of the procedure in addition to refinements of its indications and contra-indications. Moreover, an anterior cricoid split proved to be a beneficial tool in certain forms of mature subglottic stenosis. Occasionally, an anterior bilateral cricoid split is necessary, especially when the bulk of scar tissue is harboured in the lateral aspects of the cricoid ring or when the cricoid has an aberrant shape.

As splitting of the cricoid became such an important surgical technique in children undergoing laryngotracheal surgery, reports of experimental work involving different types of cricoid splits in growing animals were bound to appear. The effect of an anterior cricoid split has been comprehensively studied in young experimental animals by different authors [7-10]. The immediate gap after a cricoid split, which was observed clinically, was confirmed by all the experimental studies. The reason for this "popping open" phenomenon

of the cricoid was ascribed to the release of the interlocked stresses in the cartilage by Verwoerd et al [11], while muscle contraction working on the split parts of the anterior cricoid ring was considered by Senders et al [12] to play a role. With regard to a (bi)lateral cricoid split and a combined anterior and posterior cricoid split, a remarkable lack of experimentally documented observations exists on the behaviour of the cricoid ring after these procedures.

To investigate the morphological changes which occur in the cricoid arch after different types of splits during growth, the following series of experiments were performed in young rabbits and will be discussed in this study:

1. a unilateral cricoid split (N=10);
2. a bilateral cricoid split (N=10);
3. fragmentation of the anterior cricoid arch in four parts (N=10).

The results of a combined anterior and posterior cricoid split will be published separately.

Materials and Methods

30 young, female, New Zealand White rabbits were included in these experiments. The animals were 4 weeks old, weighing 450-600 gr, when operated upon. Anaesthesia was achieved by the administration of 1 ml/kg 10% Ketamin and 1 ml/kg 2% Xylazin-hydrochloride (Rompun).

The cricoid is approached via a midline incision in the neck through the skin, subcutaneous tissues and the strap muscles overlying the larynx.

Unilateral split (series I): the cricothyroid muscle which inserts upon the cricoid arch is mobilized laterally on the right ventrolateral side of the cricoid. The cricoid ring is interrupted by a unilateral split which is made between 1.5-2 mm from the midline.

Bilateral split (series II): on both sides of the ventral arch the cricothyroid muscle was mobilized laterally, uncovering the anterior one-third part of the cricoid ring completely. The cricoid was split bilaterally between 1.5-2 mm from the midline, creating a loose anterior fragment of the cricoid ring which measures between 3-4 mm.

Fragmentation in 4 parts (series III): after performing a bilateral cricoid split as described above, the disconnected anterior cricoid arch was divided in four equal parts.

The surgical procedures were followed by a closure of the wound in layers. Postoperatively, no antibiotics were administered. The rabbits were sacrificed at the full-grown age of 24 weeks and the larynges fixed in a formalin solution; 5μ thick sections were prepared for

histological assessment. A control series of 10 adult animals, 24 weeks of age, was available for comparison (fig.1).

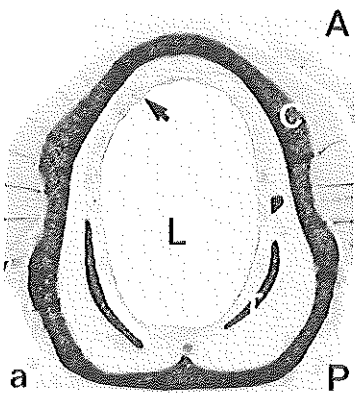

Figure 1a
Transverse histological section of normal adult cricoid. C = Cricoid, T = part of first tracheal ring, Al = Airway lumen, Arrow indicates mucosa of the airway. A = anterior, P = posterior. Magn. x6, Pas-Alcian blue staining.

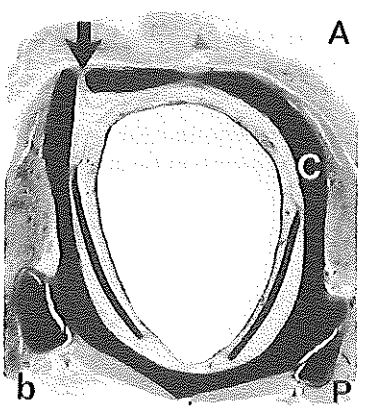

Figure 1b
Histological section of cricoid ring after a unilateral cricoid split at young age. The anterior cartilaginous arc is flattened and the latero-posterior part of the ring is deflected sidewards. No inhibition of expansion of the airway lumen. C = Cricoid, arrow indicates split. A = anterior, P = posterior.

RESULTS

All animals survived the experimental period.

UNILATERAL SPLIT (SERIES I): In 8 specimens a gap was noted between the cut ends of the cricoid ring. The size of this gap was variable (0.5 mm - 1.5 mm). In 2 specimens the cut endings of the cricoid fused again. The curvature of the ring had disappeared on the side of the dissection, the anterior cricoid arc was flattened and an outward deflection of the lateral part of the cricoid ring was evident (fig.1b). The flattening of the anterior cricoid cartilage did not induce an anterior impression of the airway lumen; no cricoid stenosis had occurred. The cut ends are rounded and covered by perichondrium. Necrosis of cartilage was not found.

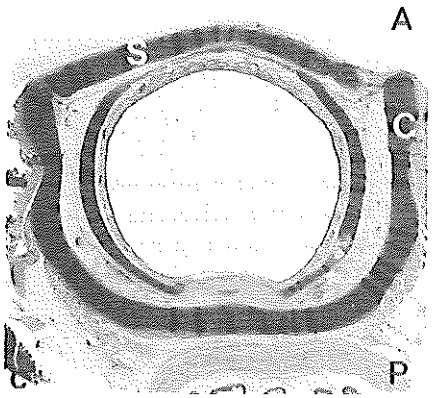

Figure 1c
Histological section through cricoid ring subjected to a bilateral cricoid split. The anterior segment is almost straight; lateral deflection of the stumps of the posterior segments. C = Cricoid, S = anterior segment, A = anterior, P = posterior.

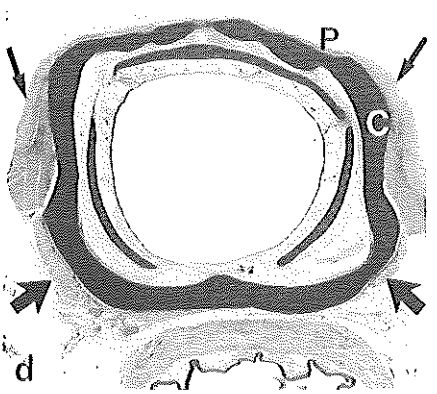

Figure 1d
Cricoid ring after segmentation of the anterior arc in four parts. The segments are stretched and connected by an outer perichondrial layer (P). Antero-posterior expansion of airway lumen is diminished C = Cricoid, Thick arrows indicate insertion of the cricopharyngeal muscle, while thin arrows indicate the cricothyroideus muscle.

BILATERAL CRICOID SPLIT (SERIES II): Flattening of the loose anterior segment was observed, causing an anterior impression of the airway. The lateral cricoid stumps demonstrated an outward deflection. It was accompanied by an increase in the transverse diameter of the posterior part of the cricoid ring (fig.1c). As a consequence, a small gap is noted between the anterior segment and the remaining cricoid ring on both sides. This cricoid configuration lead to a more circular aspect of the airway lumen. The stumps are, as was demonstrated in the specimens harbouring a unilateral cricoid split, rounded and covered by perichondrium without any sign of necrosis.

FRAGMENTATION IN 4 PARTS (SERIES III): All four fragments had lost their original curvature and were flattened. This flattening caused an anterior impression on the airway lumen as was observed in the specimens with a bilateral cricoid split. Occasionally, a cartilaginous reconnection of two segments could be observed, but in most cases the individual segments could be detected as loose fragments. Sometimes, one of the lateral fragments was connected with the posterior part of the cricoid (fig.1d) All the segments were connected to each other

(also to the large posterior segment) through an outer perichondrium. In most specimens the separate parts were not fused with each other by the inner perichondrial layer. Each individual segment was completely covered by perichondrium. Due to the lateral inclination of the remaining part of the cricoid ring, the transverse diameter had increased considerably, which is comparable to the series of the bilateral split.

DISCUSSION

Unilateral split (series I), bilateral split (series II) and fragmentation (series III) of the cricoid arch in young animals is followed by specific abnormal patterns of development, which appear to be highly constant.

The three types of malformation in these series have in common that fragments of the interrupted cricoid ring always tend to stretch and loose their curvature. This phenomenon was earlier described after an anterior cricoid split and held responsible for the occurrence of a gap between the cut edges. It is now demonstrated that stretching is not only linked to the ventral midline incision. The capacity of parts of the cricoid ring to stretch was ascribed to a release of interlocked stresses, as proposed by Verwoerd et al [11]. The concept of interlocked stresses in cartilage was introduced by Gibson [13] and Fry [14] and implied a balanced system of forces of hydrophylic proteins and collagen fibres, especially at the periphery of the structure. This concept was adapted for circular structures by adding the hypothesis that the tensile forces on the periphery of the cricoid ring exceed those on the inner side. Therefore, a separated fragment looses its curvature and shows stretching, whereas loss of the inner perichondrium on these fragments even causes a curvature to the undamaged side of the cartilage [11].

The dorsal segment of the cricoid in series II and III demonstrates an identical pattern of maldevelopment. The most dorsal part of the ring seems to be enlarged, whereas the lateral parts show a sideward inclination. At the same time the transition between the posterior and lateral part seems more abrupt than in control animals. A further difference concerns the insertion of the cricopharyngeal muscle (fig.1d), which is more laterally oriented than in the control animals. This actually suggests a sideward rotation of the lateral parts. It may be hypothesized that the action of the cricopharyngeal muscle is -besides release of interlocked stresses- a factor, causing the outward rotation of the lateral part of the interrupted cricoid ring.

In series II and III the stretching of the anterior fragments and the specific deformation of the posterior segment described above, transforms the cricoid ring in a square or even into a rectangle with the largest diameter in the transverse plane (fig.2). The ventrodorsal axis of the airway lumen is decreased. This is apparently caused by the flattening of the ventral

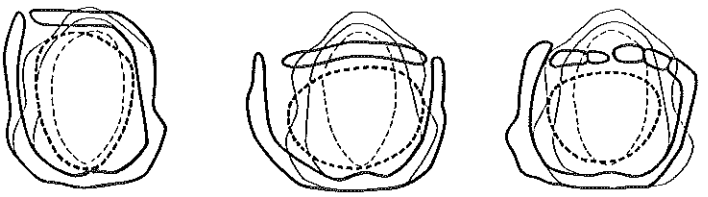

Figure 2

Schematic drawing of unilateral and bilateral cricoid split and fragmentation of the anterior arc of the cricoid ring. These schematic drawings (thick lines) are overprojected upon a normal control cricoid (thin lines) with the same magnification and summarize the deformities illustrated in Fig. 1.

part of the cricoid, which will impede the normal and ventrodorsal enlargement of the subglottic airway during growth of the curvature of the cricoid arc. Previously, it was demonstrated [7] that in absence of the anterior cricoid arch the airway lumen has a normal oval form with the largest dimension in the sagittal plane. The patency of the subglottic lumen is primarily depending on the elastic mantle surrounding and supporting the mucosa. This elastic mantle is connected to the successive segments of the laryngotracheal skeleton, in which the cricoid arch is not indispensable for maintaining the subglottic airway. An intact elastic mantle however, cannot prevent malformation of the lumen in case of abnormal growth of the injured cricoid.

Conclusions

1. Unilateral split, anterior bilateral split and fragmentation of the cricoid arc in young animals cause specific patterns of maldevelopment of the cricoid ring. One anterior cricoid split does not interfere with the antero-posterior expansion of the airway lumen, while multiple anterior cricoid splits cause a diminished antero-posterior diameter of the airway.
2. These abnormal patterns of development are ascribed to (1) release of interlocked stresses after interruption of the cricoid ring (intrinsic) and (2) the action of the cricopharyngeal muscle (extrinsic).

Further research will be done into the role of the cricopharyngeal muscle and the effect of ageing on wound healing and morphological reactions after splitting of the cricoid ring.

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THE INFLUENCE OF AGEING ON WOUND HEALING OF THE CRICOID

ABSTRACT

In young and adult rabbits the effects of one anterior and two bilateral splits of the cricoid were studied. Interruption of the cricoid ring elicits a wound healing reaction of the cut edges and induces changes in form and size of the separated parts of the cricoid (indirect effect). It was demonstrated that with increasing age: a. the wound healing capacity of the hyalin cartilage is highly diminished or lost and b. the induced remodelling involving the total ring in young animals, is in the adult stage confined to the posterior part. Moreover, the observations suggest that dividing the cricoid ring in an anterior and posterior part at a young age can result in a stimulation of growth of the anterior part in comparison with unoperated control animals.

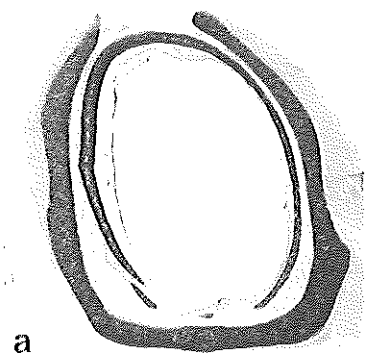
INTRODUCTION

Hyaline cartilage demonstrates remarkable changes during ageing as concluded from histological, biochemical, histochemical and electron microscopic studies [1]. Data on wound healing of hyalin cartilage in patients are only fragmentarily described [2] or limited to later stages in life [3]. Although surgery on cartilage of the nose and larynx is a common procedure at all ages and cartilage from various sites (rib, nose and ear) has been advocated as grafts, the effects of ageing upon wound healing potentials have not been reported.
In the larynx, experimental studies on wound healing are mostly confined to the subglottic level, where the development of posttraumatic stenosis is a much feared complication. Injury-specific patterns of histological and morphological reactions were identified in a series of experiments in young rabbits, after several laryngeal traumata [4,5]. Interruption of the cartilaginous cricoid ring without damage to the underlying soft tissues was demonstrated to induce permanent changes in the morphology of the fragmented ring during growth [4]. These changes are considered to be caused by interference with the interlocked stresses, present in the cartilage of the cricoid [5].
The influence of ageing on these processes and on the wound healing of the cut edges are discussed in this study. The effects of a single anterior cricoid split and anterior bilateral splits are compared during a period of growth up to the adult stage.

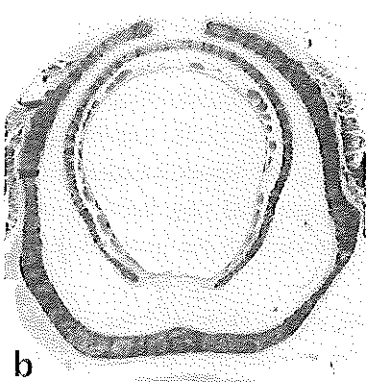
MATERIALS AND METHODS

Twenty female, 4-week-old, New Zealand White rabbits and twenty female, 24-week-old, animals were used in this study. General anaesthesia was achieved by sedation with Ketamin 10% (1 ml / kg) and Xylazin hydrochloride 2% (1 ml / kg). Both groups were divided in two series of ten animals each. In one young (Series I) and one adult (Series III) group an

anterior cricoid split was performed. A bilateral cricoid split was made in Series II and IV. The distance between the two incisions measured 3 and 5 mm in the young and adult rabbits respectively. The young animals (Series I and II) were sacrificed 20 weeks later at the adult stage. The rabbits operated upon at 24 weeks of age (Series III and IV) were kept alive for 8 weeks postoperatively. A control series (Series 0) of 10 adult animals was available for comparison. All specimens (the subglottic part of the larynx) were histologically processed to obtain 5μ thick transverse sections which were stained with a Pas-Alcian blue solution. The serial sections were studied as to the changes in size and form of the cricoid and the development of the cut edges.



(a) 20 weeks after surgery at the age of 4 weeks (Series I). Outward inclination of the cut ends.



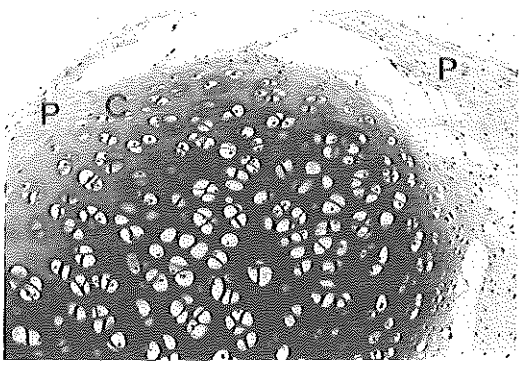
(b) 8 weeks after surgery at the age of 24 weeks (Series III). The cut ends are still positioned in the frontal plane.

Figure 1
Transverse view of the cricoid of the rabbit with an anterior cricoid split (x 6); Pas-Alcian blue staining.

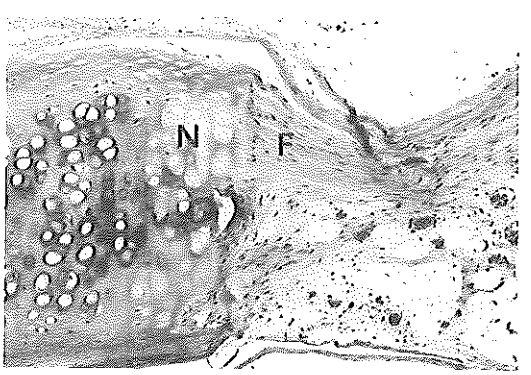
RESULTS

WOUND HEALING OF THE CUT EDGES: the cut edges in the anterior and anterolateral parts of the cricoid ring showed identical features. In series I and II the stumps were rounded and

completely covered by perichondrium (Figs. 1a and 2a). A layer of smaller chondrocytes, indicative of proliferation, separated the mature hyalin cartilage from the perichondrium. In Series III and IV in which surgery was performed in the adult stage, the level of the dissection was still prominent. (Figs. 1b, 2b). Apparently, the perichondrium had produced cells differentiating into fibrous connective tissue partially covering the stumps and forming irregular extensions, sometimes bridging the gap between the two edges. The central part of the cartilage showed an abrupt transition from viable cartilage, with a completely normal aspect, to a necrotic layer extending up to a maximum of 0.9 mm from the cut edge.



(a) 20 weeks after surgery at the age of 4 weeks. The stump is rounded and covered by perichondrium (P). Activated young chondrocytes (C).



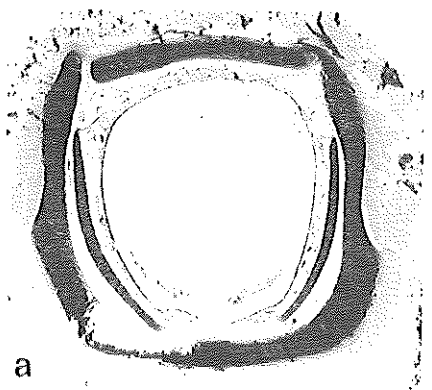
(b) 8 weeks after surgery at the age of 24 weeks. Some central necrosis (N); the viable cartilage is inert and covered by a fibrous layer (F).

Figure 2
Detailed view of the cut ends of the cricoid after splitting (x25); Pas-Alcian blue staining.

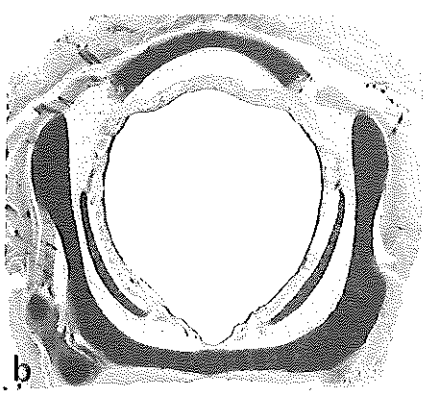
MORPHOLOGY OF THE INTERRUPTED CRICOID RING

ANTERIOR CRICOID SPLIT (SERIES I AND III): In all specimens of series I an anterior gap was present (Fig. 1a). The stumps showed an outward deflection and were positioned in a more sagittofrontal plane. In Series III (Fig. 1b) the anterior gap was smaller than in Series I; the

position of the cut ends had scarcely changed in comparison with the anterior arch in control animals and had remained in the frontal plane.



(a) 20 weeks after surgery at the age of 4 weeks (Series II). Marked flattening of the anterior part. U-like deformation of the posterior part.



(b) 8 weeks after surgery at the age of 24 weeks. Normal curvature of the anterior part. U-like deformation of the posterior part.

Figure 3
Transverse view of the cricoid of the rabbit after a bilateral cricoid split (x 6); Pas-Alcian blue staining.

BILATERAL SPLIT (SERIES II AND IV): In both series (Fig. 3a, b) the marked outward inclination of the lateral parts of the posterior fragments caused a U-like deformation.
The anterior fragments in Series II were extremely stretched and some of them seemed to exceed the "normal" length. On both sides the ends were connected to the posterior fragments (mostly) by fibrous tissues. Most of the anterior fragments in Series IV were smaller than in Series II and still showed a distinct curvature. They bridged only half the gap between the stumps of the posterior part of the cricoid.
As a result of the great variability the measurements in 10 animals did not demonstrate a significant difference. For more data the experiments will be expanded.

DISCUSSION

Interruption of the cricoid ring induces two types of reaction: First, the specific process of wound healing at the site of the injury; secondly, remodelling of the cricoid ring or its fragments.

Wound healing of hyalin cartilage has been extensively studied in the nasal septum of young rabbits (4 weeks of age). The total process took place within 2 to 4 weeks after traumatization [6]. A 0.1-0.2 mm zone adjacent to the cut edge immediately became necrotic. The underlying viable cartilage showed a reactive mitotic activity between 1 and 7 days. After two weeks the stumps were covered by perichondrium. Then, in the adult stage a small zone of neocartilage, formed in reaction to the trauma, could be discriminated by a lack of a columnar organization, as found elsewhere in the septum. In the cricoid the stumps showed a similar structure when the operation was performed at the age of 4 weeks (Series I and II). This may indicate that in young animals wound healing of hyalin cartilage follows the same pattern in the cricoid as in the nasal septum. Dissection of the cricoid in adult animals (Series III and IV) caused necrosis in a thin, most superficial zone. However, even after 8 weeks the underlying viable cartilage showed no signs of reaction. Activated cells with centrally positioned nuclei were not observed; neither mitosis nor isogenic groups of chondrocytes, produced by cell division, could be found. During maturation between the 4th and 24th week of age the hyalin cartilage seemed to loose its capacity to participate actively in the wound healing. Apparently in the adult stage two possibilities remain: the cells are lethally damaged or survive without any reaction. These observations are compatible with findings on healing between cartilage and bone ends at the costochondral junction [7]. Like in articular cartilage, where no repair can be determined [8], the adult cricoid cartilage is inert. The perichondrium, also in adult animals, remains capable of forming large amounts of cells, which differentiate into fibrous or fibrocartilaginous tissue.

With regard to the interlocked stresses in cartilage, it was hypothesized that the tensile forces on the outer side of the cricoid ring were larger than those on the inner side. Therefore interruption of the ring would result in stretching of the separated parts [5]. This stretching was seen in the posterior segments of the bilateral split in growing (Series II) and adult animals (Series IV). The anterior part in Series I and II showed definite stretching after an anterior as well as a bilateral split at a young age. In Series III and IV (adult group) stretching of the anterior part was not or minimally present. This suggests that interlocked stresses will decrease between 4 and 24 weeks after birth in the anterior part, whereas they will continue to play a role in the posterior part up to the adult age. Whether this is a reflection of regional differences in maturation remains to be investigated.

CONCLUSIONS

1. With increasing age the hyalin cartilage of the cricoid ring looses its capacity to participate actively in wound healing.
2. With increasing age the remodelling of the cricoid ring after single or multiple interruptions will progressively be confined to the posterior part.
3. The results demonstrate that effects caused by surgery or trauma lead to essential changes with increasing age. To what extent the age-related changes occur in children and adolescents remains to be investigated. These changes could be an important factor to be considered in evaluating results of treatment and planning therapy.

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CHAPTER 5

INTRINSIC AND EXTRINSIC FACTORS RELEVANT TO THE MORPHOLOGY OF THE GROWING CRICOID RING AFTER A COMBINED ANTERIOR AND POSTERIOR CRICOID SPLIT: AN EXPERIMENTAL STUDY IN RABBITS

ABSTRACT

The effects of a Rethi procedure upon the cricoid were investigated in young rabbits. An anterior and posterior cricoid split carried out upon the larynx of a young rabbit was demonstrated to result in an enlarged cricoid lumen in the adult stage due to an enhancement of both the anterior and posterior transverse diameter of the ring. These changes are ascribed to a release of interlocked stresses in the cricoid cartilage and the action of the cricopharyngeal and cricothyroid muscle.

INTRODUCTION

For many decades, surgical trauma inflicted to the soft tissues and the cartilaginous structures of the larynx of a child, was supposed to compromise the growth potential. Jackson [1] stated in 1932 that "the utmost conservatism was fundamental in dealing with the larynx of a child". It was not until 1956 that Rethi [2] pioneered the concept of a surgical expansion of the stenotic laryngeal lumen by splitting the cartilaginous skeleton. He proposed dividing the cricoid ring by a median incision anteriorly and posteriorly. Since then, several authors have reported a successful decannulation of young patients with a severe subglottic stenosis treated by a combined anterior and posterior cricoid split [3-6]. A long-term functional and radiographic evaluation of the expansion of the growing airway after decannulation, is not a routine procedure. Therefore, it is not yet known whether such a procedure will interfere in the long run with the growth potentials of the cricoid cartilage. However, Rinne et al. [7] described that 3 out of 10 children, treated with a Rethi operation, developed a gradually progressive subglottic stenosis at puberty without giving details on the cricoid.

Experimental studies concerning the effects of an anterior and posterior cricoid split upon the subglottis have been mainly restricted to adult animals [8-10]. Only Silver et al. [11] studied the effects of an anterior-posterior cricoid split in young animals during growth. They concluded that this surgical procedure does not result in a diminished growth of the cricoid. However, no data were presented relevant to the (late) effects on the morphological development of the cricoid.

This study aims to investigate the consequences of the combined anterior and posterior cricoid split for the later development of the (divided) cricoid. The larynges of growing

rabbits were chosen as an experimental model because various aspects of normal growth and wound healing of the subglottic area were previously described [12-14].

Materials and Methods

Experimental Series: 10 young (4-week-old) female New Zealand white rabbits were subjected to a modified Rethi procedure- Anterior Posterior Cricoid Split [APCS]- as described later. Their weight at the time of surgery varied from 450 to 600 grs.
The animals were anaesthetized by intramuscular administration of Ketamin 10% (1 ml/kg body weight) and Xylazin-hydrochloride 2% (1 ml/kg body weight). Postoperatively, the animals received a mixture of procain- penicillin and benzathin-penicillin (0.1 ml/kg) subcutaneously. The rabbits were sacrificed 20 weeks later, in the adult stage.

Control Series: 20 adult animals sacrificed at the age of 24 weeks.
All larynges were histologically processed; 5μ thick transverse sections were prepared and stained in a Pas-Alcian blue solution (fig.1a)
In order to compare the dimensions of the control and operated cricoids, the landmarks B, C, D and E were used (fig.1b), according to the morphometric method described earlier [12].
The morphometric data - BC, DE, the gap between the anterior stumps and the surface area enclosed by the cricoid ring (cricoid lumen) - were obtained by measuring the histological slides with a Videoplan device (Zeiss Kontron videoplan; Zeiss GMBH Oberkochen, Germany).
On both sides the most anterior and most posterior parts of the insertion of the cricopharyngeal muscle (CPM) upon the dorsolateral part of the lamina of the cricoid were chosen to define a line. The angle (CPA) between these two lines was measured in the APCS and control specimens (fig.1b).

Modified Rethi procedure (Anterior Posterior Cricoid Split [APCS])

Via a midline incision through skin and subcutis, and separation of the muscles overlying the airway, the cricoid is exposed and split in the midline.
The split creates an immediate gap as the cut edges recede promptly due to a release of interlocked stresses [15].
The airway lumen is entered via a sagittal incision through the subepithelial layer and epithelium, extending from the first tracheal ring towards the caudal edge of the thyroid cartilage. The lamina of the cricoid is reached through a translaryngeal median incision of the overlying mucosa and submucosal layer on the posterior side. This is followed by a median split of the lamina. Care was taken not to damage the underlying oesophagus. Total longitudinal splitting of the lamina was assured by momentarily pulling the two halves apart.

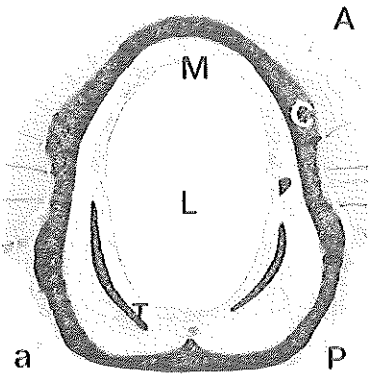


Figure 1a
Transverse histological section of the normal cricoid of an adult rabbit; magn. x6. C = Cricoid cartilage, A = anterior, P = posterior, T = part of first tracheal ring, M = mucosa, with submucosa. L = airway lumen.

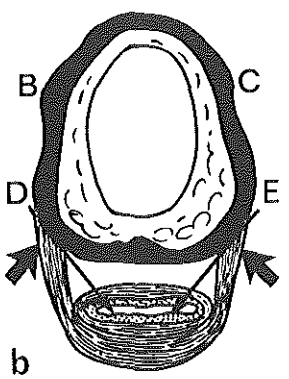


Figure 1b
Schematic drawing of fig.1a. B and C are landmarks which indicate the transition between the anterior cricoid arch and the lateral parts of the cricoid ring. D and E indicate the transitional zone between the lateral parts of cricoid ring and lamina of the cricoid. The insertion of the cricopharyngeal muscle upon the dorsolateral side of the cricoid ring is indicated (bold arrows). Along these insertion areas two oblique arrows which create an angle, are drawn.

No stents were used. The cervical wound was closed in layers with absorbable sutures.

RESULTS

MORPHOLOGICAL OBSERVATIONS

In all specimens of the APCS series, the anterior cricoid gap is widely open (fig.2 and 3). The anterior cricoid stumps are rounded and covered with perichondrium. Apposition of new cartilage by this perichondrium is a regular finding (fig.4a). There are no signs of necrosis. Collagen fibers can often be observed between both anterior stumps (fig.2).

Posteriorly, the cut edges of the lamina are approximated and reconnected by fibers or fibrocartilaginous tissue or newly formed hyalin cartilage (fig.4b). A slight overlap of the edges was found in 3 specimens (fig.3).

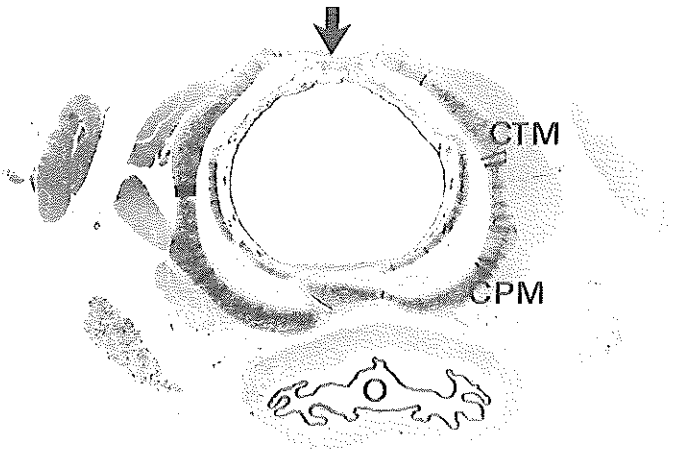


Figure 2

Histological section of adult cricoid, 20 weeks after an anterior-posterior cricoid split. Anterior gap with collagen fibers in between (arrow) and posterior (fibrous) fusion with slight overlap of the posterior stumps. CPM = insertion of cricopharyngeal muscle upon cricoid, CTM = insertion of cricothyroid muscle. O = oesophagus with constrictor muscle.

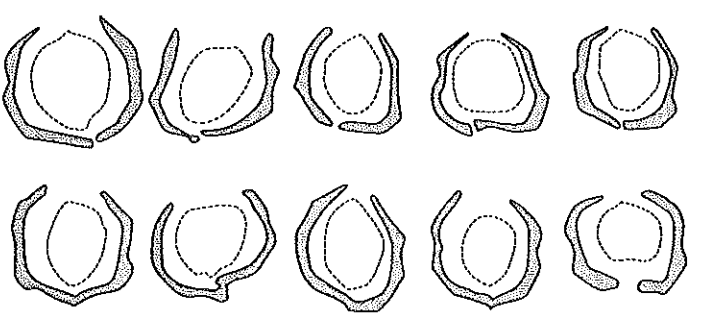
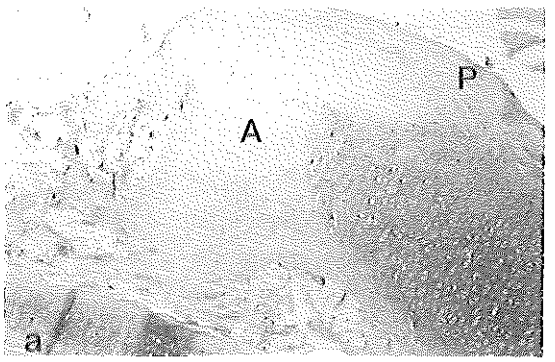


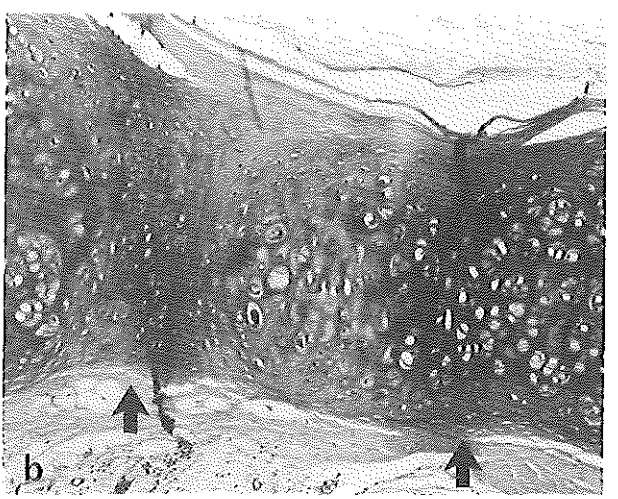
Figure 3

Schematic drawing of all cricoids in APCS series. Anterior and posterior widening of the cricoid ring is observed in all specimens. Lumen of the cricoid ring has changed and has a more circular configuration.

The morphology of the separated halves of the cricoid ring is always abnormal and shows a marked individual variation (fig.3). Most of the cricoid segments demonstrate a degree of stretching compared to their normal curvature. The anterior endings are outwardly rotated, contributing to an enlargement of the anterior gap. Finally, the specific pattern of thinner and thicker areas of the cricoid ring, a constant finding in control animals, appeared to be irregular or even absent in the APCS series.



a: Thickening of the anterior stump by newly formed cartilage. P = perichondrium, A = appositional (newly formed) cartilage.



b: cartilaginous fusion of the cut edges of the lamina; arrows indicate the dissection edges of lamina.

Figure 4
Detailed view of anterior stump (a) and posterior fusion (b).

At the site of the anterior and posterior incision through the soft tissues lining of the airway, the mucosa has completely regenerated and is covered by a normal ciliated cylindrical epithilium. The subepithelial layer might be slightly thickened as a result of the deposition of fibrous tissue and occasionally ectopic cartilage. Inflammatory cells can not be found at this stage.

In 8 out of the 10 specimens the first tracheal ring is almost completely present in sections at the level of the cricoid ring, and in some cases it appeared that this first tracheal ring had been transsected.

Morphometric Observations

For the anterior gap an average of 4.39 mm was measured (table 1). Compared to the adult control series, the cricoids in the APCS group show an increased transverse diameter at the levels B-C and D-E. The area enclosed by the interrupted cricoid ring is larger in the APCS group (47.78 sq mm/ s.d.7.24) than in the control group (32.68 sq.mm/s.d. 3.80).
The angle (CPA) determined by the lines through the insertion of the cricopharyngeal muscle on both sides of the cricoid (fig.1b) appeared to be significantly larger in the APCS series than in the control specimens.

Table 1
*Mean values of the cricoid lumen and the transversal diameter (B-C, D-E), after a combined anterior and posterior cricoid split. * = significantly different with $p < 0.05$. Mann-Whitney test. SD=standard deviation.*

	cricoid lumen mm^2	gap mm	B-C mm	D-E mm	angle (CPA) degrees
Control	32.68 sd 3.80	-	5.19 sd=.43	6.07 sd=.39	60° sd=10
APCS	47.78 * SD=7.24	4.39 SD=1.61	7.06 * SD=.87	7.43 * SD=.73	93° * SD=20

Discussion

An anterior and posterior cricoid split (APCS) is demonstrated to induce in young rabbits a specific pattern of abnormal development of the divided cricoid ring, characterized by:

1. a large persistent gap between the anterior stumps of the cricoid.
2. reconnection of the cut edges of the lamina (posterior ends) in most of the specimens.
3. stretching of the separated cricoid fragments.
4. outward rotation of the cricoid halves (increased CPA, BC and DE).

These changes result in an increased cricoid lumen cross sectional area.
Modifications of the morphological development of the cricoid ring as the result of a single or multiple split have been reported previously [13]. An essential feature of the growing fragments of the cricoid was demonstrated to be a diminished curving of the cartilage. This has been ascribed to the release of interlocked stresses in the cartilage, which are thought to

be stronger at the outer than at the inner margin of the cricoid ring [15]. In this respect the longitudinal halves after an APCS react similar to the anterior and posterior segments after a bilateral split [15]. On the other hand, release of interlocked stresses can not explain the outward rotation of the cricoid halves with a persistence of the anterior gap and reconnection of the posterior stumps. Therefore, the question arises which extrinsic forces could influence the cricoid fragments.

The cricoid is suspended in a three-dimensional system of muscles, of which the cricopharyngeal muscle (CPM) and the cricothyroid muscle (CTM) are the most conspicuous. The CPM inserts upon the dorsolateral part of the lamina on both sides and surrounds the pharynx. Contraction of the CPM during swallowing results in a pulling force at the sites of insertion (fig.5).

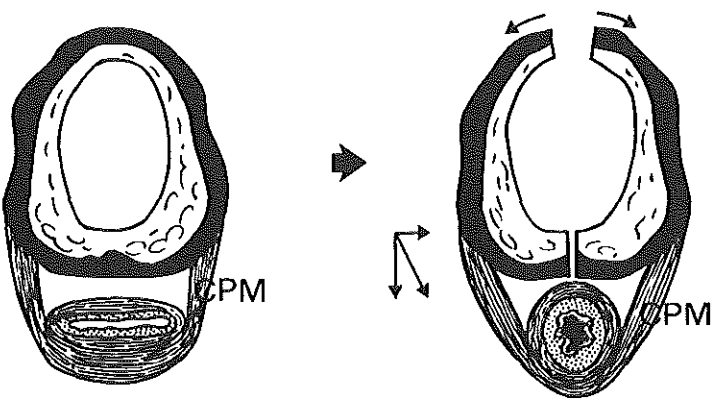


Figure 5
Schematic drawing of the cricopharyngeal muscle (CPM) and the insertion upon the cricoid ring as seen in the transverse plane. The contracting CPM exerts a tilting force upon the lateral halves of the cricoid, divided by an APCS.

After an APCS this could lead to reapproximation of the posterior cut edges, an outward rotation of the mobile cricoid halves and an enlargement of the anterior gap. As these phenomena are actually observed in the APCS series it may be hypothesized that the action of the cricopharyngeal muscle contributes to the morphological changes of the longitudinally split cricoid ring.

The insertion of the CTM is found upon the cricoid arch and the anterior edge of the cricoid lamina. Its major action comprises an elevation of the cricoid ring. A relatively small component of this muscle action could be involved in a lateral displacement of the separated cricoid halves [16]. Further experimental analysis is needed to elucidate the influence of various laryngeal muscles on cricoid pathology.

It can be concluded, however, that morphologic changes of the cricoid after an APCS are the result of an intrinsic factor (release of interlocked stresses in the cartilage) and an extrinsic factor (muscle action). Splitting of the cricoid means interruption of the ring and interference with the balanced forces of the muscular system in which the cricoid is suspended, both with consequences for the future morphologic development.

Previous authors [9,11], studying various splits in animal experiments, did not comment on the morphologic changes of the cricoid, as reported here. They focused on the dimensions of the subglottic airway [9] and the cross-sectional area of the cartilage of the cricoid ring. Strome et al. [10] and Zalzal et al. [17] did report an unstable apposition of the posterior stumps in adult dogs contrary to the fibrous and cartilaginous connections in our experiments. This difference could be related to a difference in species (dog-rabbit) or age (young-adult) at the time of surgery. Recently, a decrease of wound healing capacities of the cricoid cartilage was demonstrated with increase in age [18]. This could explain the lesser stability of the posterior edges in adult experimental animals compared to young animals. The longitudinal incisions in the soft tissue lining both posteriorly and anteriorly showed in our series an excellent wound healing without the formation of a stenosis. Prolonged inflammatory processes seem the most probable reason for scar formation and a soft tissue stenosis [14]. To what extent the increase of the cricoid "lumen" results in an increase of the subglottic airway can not be concluded from these data at hand as the cross-sectional airway lumen at the level of the cricoid appeared to be defined in most specimens by the first tracheal ring, trapped within the cricoid. It is possible that widening of the cricoid lumen promotes this upward displacement (trapping) of the first tracheal ring.

CONCLUSIONS

1. A combined anterior-posterior cricoid split in young rabbits results in a specific pattern of maldevelopment of the cricoid ring.
2. The anterior gap after a combined anterior-posterior cricoid split is larger compared to the gap after a solitary anterior cricoid split because of the tilting action of the cricopharyngeal muscle upon the posterior part of the cricoid, and the release of interlocked stresses in the cricoid segments.

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CHAPTER 6

INJURY- AND AGE-LINKED DIFFERENCES IN WOUND HEALING AND STENOSIS FORMATION OF THE SUBGLOTTIS

ABSTRACT

The effects of superficial and deep endolaryngeal trauma of the subglottic airway were studied in young and adult rabbits. In both age groups a soft stenosis was formed as long as the cartilaginous cricoid ring was not involved. This stenosis comprised a thickened subepithelial zone of scar tissue, separated from the cricoid cartilage by a layer of fatty tissue. Injury of the internal side of the cricoid cartilage induced a compact mass of scar tissue with local differentiation into fibrocartilage. In young animals only, injury of the cartilage led to remodelling of the cricoid ring (indentation or collapse of the traumatized sectors). On the basis of the differentiating effects of age and depth of the lesion, three histopathological types of subglottic stenosis were distinguished. The experimental results provide an explanation for the variability in the histopathological features of the wall of the stenotic subglottic airway, as observed in biopsies and post-mortem specimens.

INTRODUCTION

The histological identification of the precise nature of a subglottic stenosis has been the subject of several studies of human specimens [1-5] and larynges of experimental animals [6-11]. From these studies it was concluded that subglottic stenosis comprises a heterogenous group of histopathological conditions. An initial investigation of the cause of this heterogeneity in young rabbits was made by Adriaansen et al.[11-13]. The morphological features of induced subglottic stenosis were demonstrated to be related to the depth of the initial endolaryngeal lesion. These authors investigated endolaryngeal injury with or without involvement of the cricoid cartilage. The latter condition results in a soft tissue stenosis, mainly composed of a subepithelial layer of fibrous scar tissue. Endolaryngeal trauma involving the cartilage gives rise to a compact stenosis, characterized by the formation of a large amount of scar tissue combined with a progressive deformation (irregular collapse) of the cricoid ring.

A study of wound healing after cricoid splits showed that the ability of cartilage to participate in regenerative processes, is highly influenced by increasing age [14]. Therefore, it was hypothesized that - besides the depth of the injury - age-related differences in wound healing could be another factor, responsible for the histological variability of subglottic stenosis developing after endolaryngeal trauma. To test this hypothesis we repeated our earlier experiments [11,13] in adult rabbits.

Material and Methods

Twenty adult female New Zealand White rabbits (weighing between 3500 and 4500 gr) were used in these experiments. The experimental animals were anaesthesized by intramuscular administration of Ketamin (1 ml/kg) [Bayer] and Xylazin-hydrochloride (1 ml/kg) [Apharma].

Series A: Superficial subglottic endolaryngeal trauma (N = 10)

The larynx and trachea were exposed via a midline incision through the skin and subcutaneous tissue, followed by separation of the pre-laryngeal and pre-tracheal muscles. A transverse incision was made through the cricotracheal ligament into the airway lumen. Through this opening the epithelium and subepithelial layer overlying the cricoid ring were circumferentially traumatized with a thin burr. The perichondrium was left undisturbed. The gap between the first tracheal ring and the cricoid ring was closed with absorbable sutures. The overlying muscles, subcutaneous tissue and skin were closed in layers.

Series B: Deep subglottic endolaryngeal trauma (N = 10)

The subglottic lumen was exposed in the same way as described above. In addition to circumferential removal of the soft tissues, the perichondrium and subperichondrial cartilage of the cricoid are injured with the drill. The wound was closed in a similar way as in series A.

The experiments were finished when (initial) wound healing was assumed to be completed, 8 weeks after the endolaryngeal trauma [15]. However, 4 rabbits of group B were killed after respectively 2,3 (two animals) and 4 weeks because of an apparent dyspnea. All animals in series A survived the 8 week postsurgical period. The larynges were harvested, fixed in a 10% formaldehyde solution and processed for histological studies. 5μ thick sections were obtained and stained with a PAS-Alcian blue solution. In addition representative sections of 5 specimens were stained with a R(esorcin) F(uchsin) solution (RF) to assess the condition of the elastic fibers of the tunica elastica.

Control series

The histological sections of larynges of 20 unoperated adult female New Zealand White rabbits were available for comparison. These sections were made and histologically processed with the same procedure described above for the experimental animals.

Morphometric measurements of the airway lumen were performed by tracing a standard magnified projection of a representative transverse histological section of each cricoid. Every measurement was introduced into the computer and the actual size of the airway lumen was calculated [Zeiss Kontron Videoplan; Zeiss, GMBH Oberkochen, Germany].

RESULTS

MORPHOMETRIC DATA

Measurements of the airway lumen in series A and B specimens showed that the mean surface area of the subglottic lumen was 17.83 sq.mm and 12.66 sq.mm respectively, compared to a mean surface area of 21.1 sq.mm in the control group. Slight to moderate stenosis had therefore developed after superficial subglottic endolaryngeal trauma, whereas deep subglottic endolaryngeal trauma resulted in a severe stenosis (table I).

Table I

Measurements of the mean subglottic airway lumen 8 weeks after a superficial (group A) or deep (group B) endolaryngeal trauma compared to the dimensions of a normal control cricoid. For both groups a significantly decreased airway lumen is observed. Mann-Whitney test. SD= standard deviation.

	control	group A (SSET)	group B (DSET)
mean airway lumen mm^2	21.1 SD=2.9	17.83 SD=2.15 $p<0.05$	12.66 SD=4.20 $p<0.01$

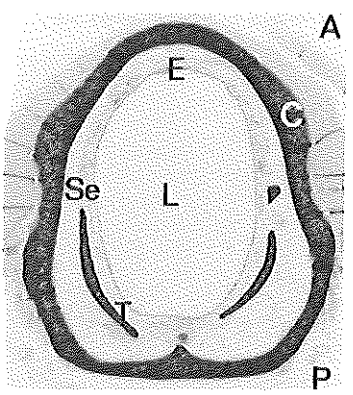
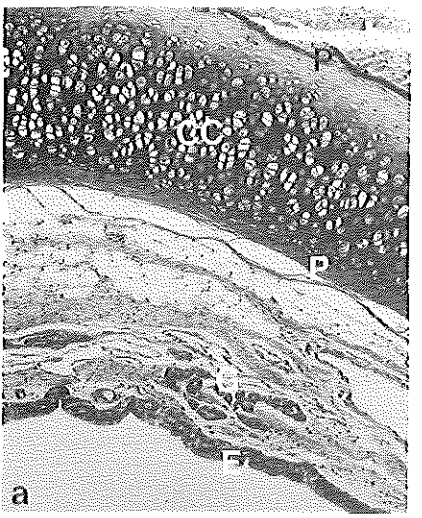


Figure 1
Transverse histological section of the cricoid of an adult rabbit (control). Magn. x6, Pas-Alcian blue staining.
C = cricoid cartilage
A=anterior
P=posterior
E=epithelium
Se=subepithelial layer
T=part of first tracheal ring
L=airway lumen

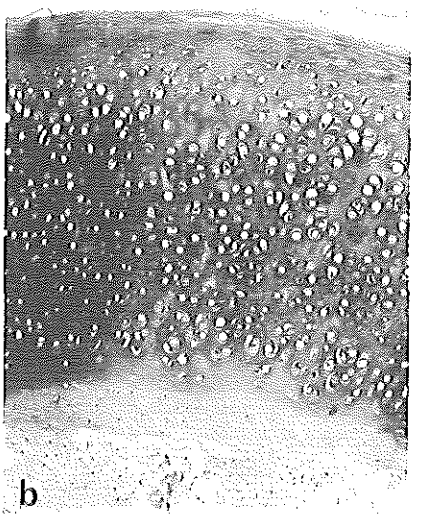
HISTOLOGIC OBSERVATIONS

Control series: The wall of the airway at the subglottic level consisted of soft tissues lining the lumen and the cricoid ring (fig.1). The soft tissue lining was composed of an epithelium and a subepithelial layer, containing mucous glands (fig.2a). Both epithelium and glands were supported by a surrounding mantle of elastic fibers (tunica elastica). At the periphery adjacent to the cricoid ring, mainly loose connective tissue with fatty cells were found. The cricoid ring showed a core of mature hyalin cartilage. In section this core was covered by perichondrium on the internal and external sides of the ring (fig.2a).

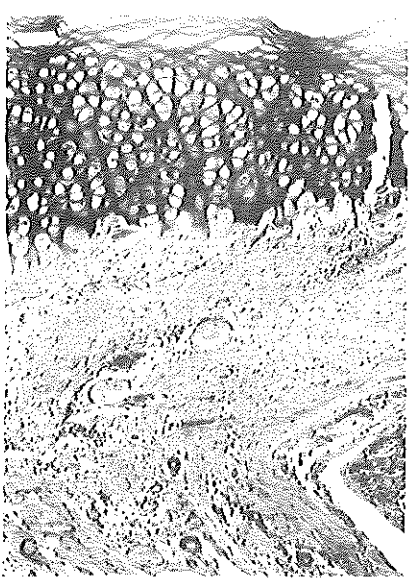
SERIES A: The epithelial lining was complete in all specimens, and varied from normal cubic ciliated epithelium to a flat or multilayered epithelium. In all specimens, thickening of the



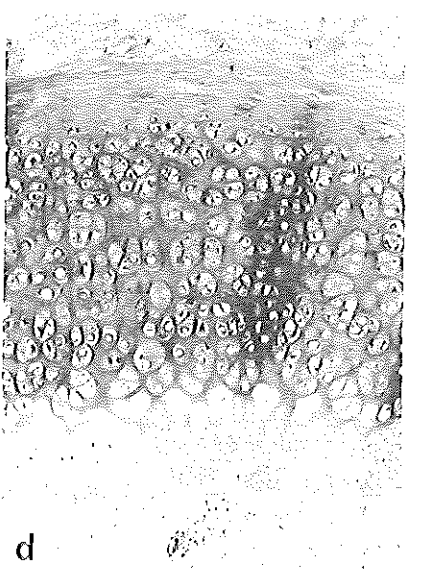
2a. Control series; CC=core of cricoid, P=perichondrium, E=epithelium, G=mucous glands. Magn. x10



2b. Deep trauma, type I; loss of perichondrium; chondroneogenesis at the internal surface of cricoid. Magn. x25.



2c. Deep trauma, type II; disintegration of core of cricoid.



2d. Deep trauma, type III; regressive changes in the core of cartilage; neocartilage at the external side.

Figure 2

Details of transverse histologic sections of the cricoid in higher magnification. Pas-Alcian blue.

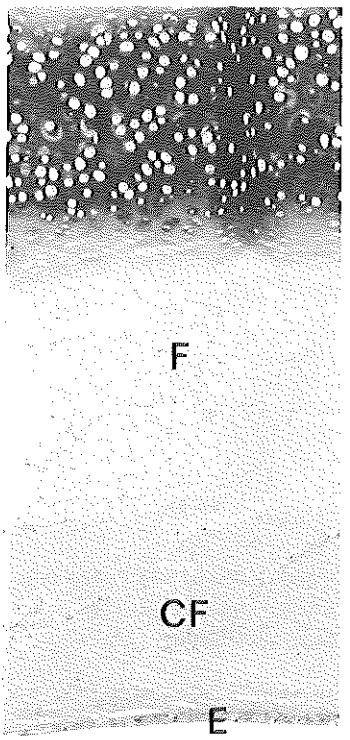


Figure 3
Superficial endolaryngeal trauma (series A). Airway lumen covered by ciliated epithelium (E); bundles of collagen fibers (CF) deposited directly underneath the epithelium; fatty tissue (F) between cricoid ring and fibrous scar tissue. Mucous glands are absent. Magn. x25, Pas-Alcian blue.

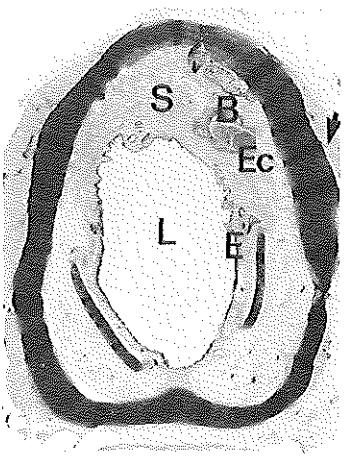


Figure 4
Transverse section of the cricoid ring 8 weeks after a deep subglottic endolaryngeal trauma (Series B). Scar tissue (S) anteriorly, which is highly vascularized; ectopic cartilage (Ec) and bone (B); finger-like projections of the epithelium (E) into the airway lumen (L); extra thickening of cricoid - typeIV (arrow). Magn. x6, Pas-Alcian blue.

subepithelial layer caused a narrowing of the subglottic lumen. This was characterized by fibrous scar tissue with a circular orientation, mainly located immediately under the epithelium. Separating the scar tissue and the cricoid ring, a zone of loose connective tissue and fatty cells was often observed (fig.3). Inflammatory cells were detected sporadically. Neither ectopic cartilage nor bone was found in the scar tissue. In RF-stained sections, the three-dimensional arrangement of the elastic fibers appeared to be completely lost in the stenotic area, as only fragments of elastic fibers could be recognized. The cricoid cartilage did not show any noteworthy changes.

SERIES B: The airway lumen was lined by a polymorph epithelium, varying from ciliated cubic epithelium to flat or multilayered. In the airway lumen, numerous villiform projections

covered by epithelium were observed (fig.4). The subepithelial layer manifested varying degrees of thickening due to the formation of dense scar tissue. This contained many small blood vessels, areas of fibrocartilage, islands of bone with a central marrow cavity, a few scattered elastic fibers and cystic structures lined with ciliated epithelium. This dense mass extended from epithelium to cricoid ring. In particular, the larynges of the 4 animals which were sacrificed 2 to 4 weeks after the endolaryngeal trauma, showed severe subglottic stenosis, with a pin-point lumen, located eccentrically and more posteriorly (fig.5). In all specimens, most of the scar tissue was situated on the anterior side.

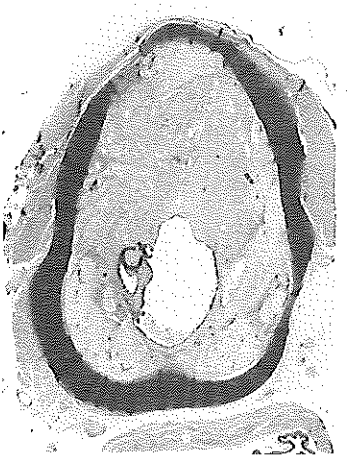


Figure 5
Transverse histologic section of cricoid ring 3 weeks after a deep endolaryngeal trauma. Massive scar tissue, mainly anteriorly; pin-point lumen; cystic structure (Ct). Magn. x6, Pas-Alcian blue.

The injured cricoid cartilage showed different reactions along the internal surface. Basically, the following types of reactions could be distinguished:

I. Various degrees of thickening due to chondroneogenesis at the internal surface of the cricoid ring (fig.2b)

II. Loss of internal perichondrium and underlying cartilage; the remaining mature cartilage of the central zone showed signs of disintegration without formation of new cartilage and was covered by fibrous scar tissue (fig.2c)

III. Loss of the internal perichondrium and regressive changes in the core of cartilage, together with the formation of new hyalin cartilage on the external surface of the cricoid ring (fig.2d)

In some specimens sectors of the cricoid ring showed an extra thickening (reaction type IV) (fig.4)

The reactions summarized above could appear simultaneously at different sites of a single cricoid ring. Collapse or remodelling of the ring as previously reported in young animals [11] was not observed in the adult animals studied here.

DISCUSSION

Striking similarities and differences can be noted when wound healing processes and the subsequent formation of subglottic stenosis are compared between young rabbits [11,13] and adult rabbits (series A and B). The sequelae of superficial endolaryngeal trauma (restricted to the soft tissue lining of the subglottic airway) are very similar in both age groups. Slight or moderate stenosis is the final result of a thickened subepithelial layer, consisting of a zone of scar tissue with circularly orientated fibers, separated from the normal cricoid by a zone of fatty tissue. A difference is that -especially in the young animals- the scar tissue includes islands of ectopic cartilage.

On the other hand, the effects of a deep endolaryngeal trauma involving the soft tissue lining and cartilage of the cricoid in both age groups show major differences.

In both young and adult animals the result is a severe stenosis. The subepithelial layer is thickened by a compact mass of dense collagenous scar tissue with local differentiation of fibrocartilage. In the adult stage (series B) the scar tissue presents islands of bone, and cysts lined with ciliated respiratory epithelium and filled with mucus.

The major age-related differences in wound reaction concern the cricoid cartilage. First, the young cricoid responds to injury with local necrosis followed by restoration of the morphological features due to the chondrogenic potential of the young perichondrium and cartilage [12]. In the adult animal, the perichondrium and subperichondrial cartilage apparently form some new cartilage, but the central mature, most differentiated cartilage shows only signs of disintegration and necrosis. This confirms earlier observations on the various processes of wound healing of cut edges after cricoid splits in young and adult animals [14]. The other major difference is in the morphology of the cricoid ring. In adult animals the ring-like morphology is maintained in all specimens. However, identical endolaryngeal trauma in young animals involving the internal surface of the cricoid invariably results in remodelling of the cricoid ring with irregular indentation (inward collapse) of segments of the ring. In a previous study this remodelling was attributed to release of interlocked stresses due to traumatic injury of the perichondrium on the internal side of the ring [16]. Apparently, this mechanism does not play a role in the adult stage. Histological maturation of the cartilage of the cricoid between 4 and 24 weeks (the adult stage) is probably reflected as the loss of regenerative potential of the central cartilage and the loss of reactive remodelling of the cricoid ring.

The classification of subglottic stenosis as proposed by Holinger [17] summarizes histopathological observations. However, on the basis of pathogenic features and in particular, the differentiating effect of the depth of the endolaryngeal injury and the age (as demonstrated in the animal experiments) we distinguish 3 histopathological types of subglottic stenosis:

A. Soft tissue stenosis - superficial trauma (young and adult)
B. Compact stenosis without remodelling of the cricoid ring - deep trauma (adult)
C. Compact stenosis with remodelling of the cricoid ring - deep trauma (young)

With respect to the observations in patients with subglottic stenosis, chronic inflammation of the inner perichondrial layer and cricoid cartilage is often mentioned as the cause of thickening of the cricoid ring after endolaryngeal trauma [18,19]. In the specimens we studied however, no signs of florid perichondritis were seen. On the other hand, it seems to be the injured perichondrium, triggered to produce large numbers of new cells, which is responsible for the compact character of the stenosis. Secondly, cartilage encountered in a subglottic stenosis is sometimes described as hamartoma; consistent observations in both growing [20] and adult animals (series B) suggest that cartilage and bone formation can represent a "normal" phenomenon in the wound healing process of subglottic tissues.
Finally, subglottic cysts are sometimes noticed in the larynx of patients who were intubated for a longer period of time [21,22]. It has been suggested that the formation of these cysts is caused by the occlusion of glandular ducts [21]. However, the ciliated epithelial lining of the cysts as observed in this study, suggests that an inclusion of (mucus producing) respiratory epithelial cells is much more likely.

CONCLUSIONS

1. The heterogeneity of the histological features of the wall of the stenotic subglottic airway as concluded from biopsies and post-mortem specimens, is the result of
   a. age-linked differences in wound healing of the injured cartilage
   b. age-linked differences concerning the reactive remodelling of the cricoid ring
   c. differences in the wound reaction related to the depth of the endolaryngeal injury
2. Identical endolaryngeal lesions will induce different reactions with increasing age
3. Even slight differences in the depth of the injury along the circumference of the subglottic airway contribute to the histological heterogeneity of the resulting stenosis.

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CHAPTER 7

CHONDRONEOGENESIS IN A COLLAGEN MATRIX

ABSTRACT

In this pilot study, demineralized bovine bone matrix was implanted in a vascularized perichondrial layer of the ear cartilage in young rabbits and histologically assessed after respectively one, two and three weeks. It appeared that the bone matrix is gradually replaced by autologous cartilage tissue. Subsequently, the resulting cartilage graft was used to reconstruct the anterior arch of the cricoid cartilage in growing rabbits. 20 weeks after the reconstruction procedure, the graft was solidly attached to the cricoid cartilage and did not induce a subglottic stenosis, as apparently the graft had grown commensurately with the cricoid ring.

INTRODUCTION

Senn [1] successfully used demineralized allogenic bone matrix as a substitute for fresh autologous bone grafts to reconstruct bony defects in dogs and patients. Various animal experiments have shown that decalcified bone matrix grafts, implanted in skeletal or extraskeletal sites, undergo resorption and are replaced by new bone, often through a process of enchondral ossification [2-5].

Perichondrium is a well-known potential source of cells, capable of chondrogenesis [6,7]. However, this process appeared to be absolutely unpredictable when a free perichondrial graft was used. This is a disadvantage for clinical use.

The first aim of this study was to investigate whether a piece of bone matrix, predetermined in size and shape could be transformed into a cartilaginous structure by exposing this material to a vascularized perichondrial flap.

The second aim was to study the feasibility of such a structure as a graft to reconstruct a growing defective cricoid.

MATERIALS AND METHODS

Twelve 4-week-old rabbits (New Zealand White, female) were divided into four groups of three animals each. Anaesthesia was accomplished by administering Ketamin (1 ml/kg IM) and Rompun (1 ml/kg IM). After incision of the skin of the anterior surface of the left ear a pedicled perichondrial flap was elevated from the ear cartilage. A piece of demineralized bovine bone matrix (Osteovit ®) measuring 10x7x2mm, was wrapped in the perichondrial flap before suturing the skin incision. The demineralized bone was left in situ for 7 days (group 1), 14 days (group 2), 21 days (group 3 and 4) respectively. The grafts, harvested in the groups 1, 2 and 3 were fixed in a 10% Formaldehyde solution and histologically processed; 10μ thick sections were prepared for Pas-Alcian blue and Von Giesson staining.

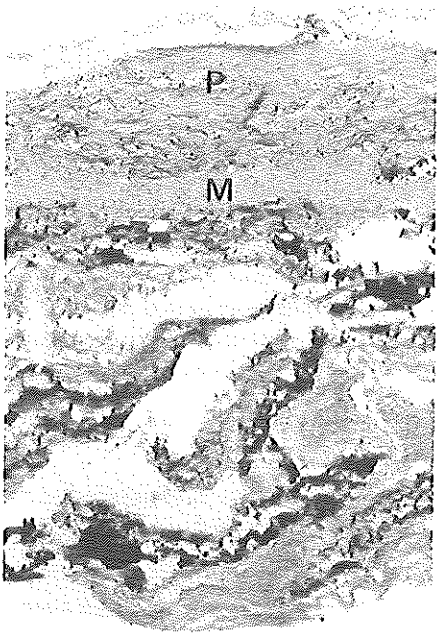

Figure 1
Histological section of DBBM after implantation in a vascularized ear perichondrial pocket for 1 week. Perichondrial layer (P) is swollen and just beneath this layer a mesenchymal cell layer (M) can be detected leaving the perichondrium (x10).

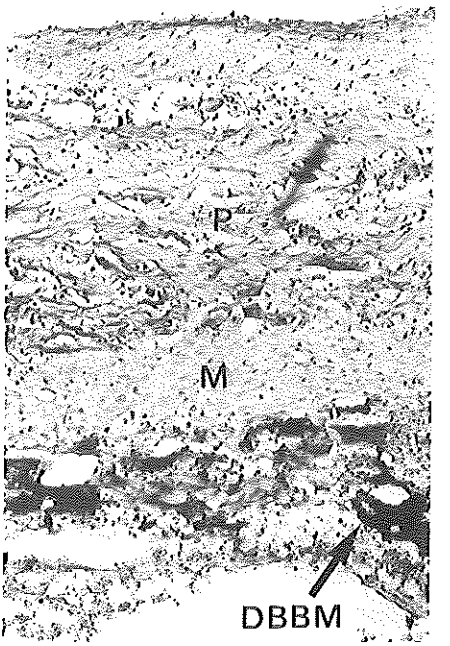

Figure 2
Detailed view of window in fig.1. Superficial layer of bone matrix (DBBM) contains ballooned mesenchymal cells (M). Perichondrium (P) is extremely swollen, with large spaces between collagen fibers. Perichondrial cells are rounded with swollen nuclei. (x 25)

The grafts of the animals in group 4 were used for the reconstruction of the cricoid in the same animals. The cricoid was exposed through a mid-line incision. The anterior 1/3 part of the cricoid was resected and replaced by an arc-shaped piece of the cartilaginized matrix. The remaining parts of the latter were studied histologically. Twenty weeks later the adult rabbits of group 4 were sacrificed; the larynges were processed and 10μ thick transverse sections were stained in a Pas-Alcian blue procedure.

Results

Matrix-perichondrium

After 1 week the demineralized bone matrix-perichondrium graft is very fragile and the interstices of the matrix are mainly occupied by blood clots. The perichondrium is very loosely attached to the matrix.

In histological sections the perichondrium appears to be extremely swollen (fig.1). The collagenic bundles are separated by edema. The perichondrial cells are swollen with rounded

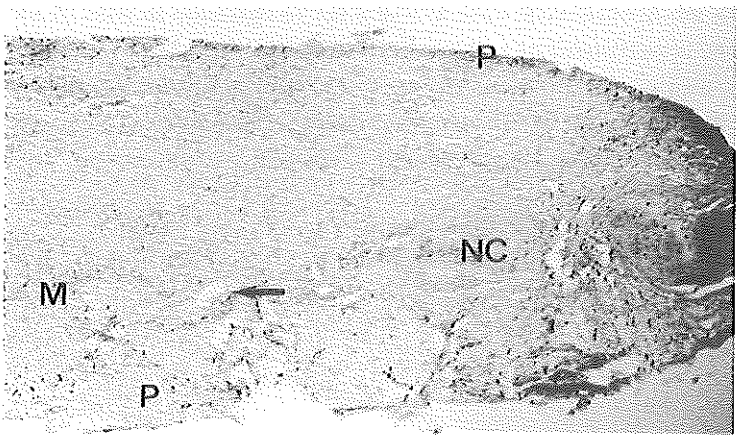

Figure 3

Demineralized bovine bone matrix colonized by mesenchymal cells and cartilage after a contact period of two weeks with the vascularized perichondrial layer. Numerous blood vessels are observed (arrow); PAS - Alcian blue staining. NC= islands of neocartilage, P= perichondrium, M= mesenchymal cells. Original magnifation x 10.

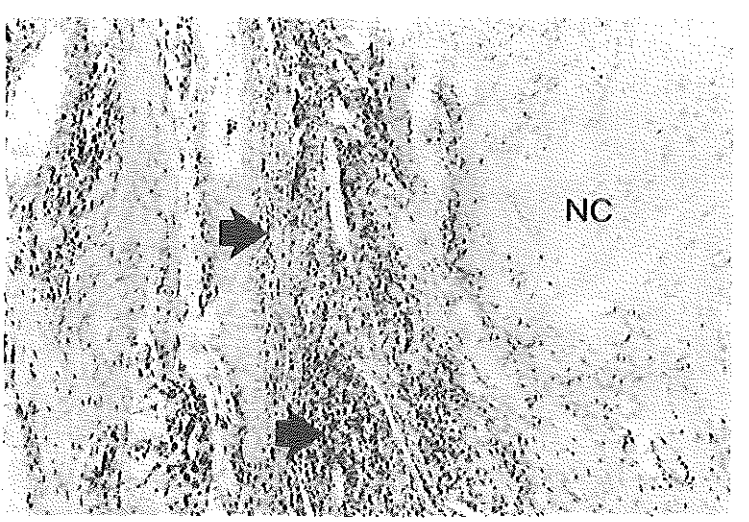

Figure 4

Detailed view of former demineralized bone matrix implant 3 weeks after implantation with large areas of neocartilage (NC). Islands of mesenchymal cells intermingled with inflammatory cells are still present (arrow). PAS - Alcian blue staining. Original magnification x 25.

nuclei. These cells with a mesenchymal aspect are also found in large quantities between the perichondrium and the bone matrix (fig.2). They even invade the superficial layer of the latter.

After 2 weeks the demineralized bone matrix is not recognizable as such. It shows a solid consistency and is surrounded by a tightly fitting perichondrium. In histological sections the perichondrial layer is still thickened. The mesenchymal cells have completely colonized the bone matrix (fig.3). Islands of newly formed cartilage, numerous bloodvessels and many inflammatory cells are found. Only a remnant of the matrix is still present.

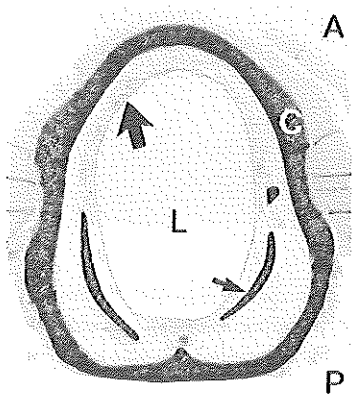

Figure 5
Normal cricoid of adult rabbit (24 weeks) with part of first tracheal ring (thin arrow) in subepithelial layer, covered by epithelium (thick arrow); PAS - Alcian blue staining. C = cricoid, L = airway lumen, A = anterior, P = posterior. Original magnification x 6.

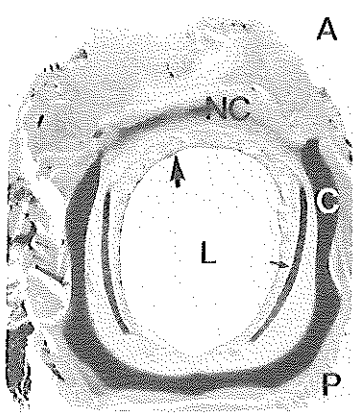

Figure 6
Cricoid of an adult rabbit (24 weeks) reconstructed with a cartilaginized bovine bone matrix implant at the age of 7 weeks. The upper part of the first tracheal ring (thin arrow) lies in the subepithelial layer, covered by epithelium (thick arrow). PAS - Alcian blue staining. NC = arc shaped neocartilage implant fused with host cricoid cartilage, C = cricoid, L = airway lumen, A = anterior, P = posterior. Original magnification x 6.

After 3 weeks the implant is firm and macroscopically resembles cartilage. Histological investigation demonstrates that the bone matrix is almost completely resorbed and replaced by cartilage (fig.4). Locally, small areas of mesenchymal cells intermingled with inflammatory cells can still be distinguished. There is no bone formation.

CARTILAGINIZED MATRIX GRAFTS IN THE CRICOID

Twenty weeks after the cricoid reconstruction endoscopy revealed no airway stenosis. On histological assessment the subglottic lumen displayed no reduction of the surface area in comparison to the normal cricoid (fig.5). In the reconstructed cricoid an arch of newly formed cartilage, surrounded by perichondrium is found (fig.6). The cartilage appeared to be mainly of the hyaline type, but also areas of fibrocartilage are present. The graft-cricoid connections are cartilaginous.

Small areas of bony tissue, located around a bloodsinus or marrow cavity are a regular finding in these specimens.

DISCUSSION

Demineralized bone matrix is known to be transformed into bone through a process of colonization by mesenchymal cells and subsequently, differentiation into cartilage and finally into bony tissue [8]. Ossification was reported to be completed approximately 3 weeks after implantation of bone matrix in extraskeletal sites [9].

In this first study of bone matrix in contact with perichondrium the high chondrogenic activity of the perichondrium was demonstrated within 2 weeks, resulting in an almost complete cartilaginization of the matrix after 3 weeks without any sign of ossification. Only 20 weeks later was some bone formation observed in grafts taken from the cartilaginized matrix. Besides mesenchymal cells many inflammatory cells initially invaded the allogenic material of the matrix. Different degrees of inflammatory reaction, described in several reports [10] may be due to the different sources of the bone matrix (homologous, autologous or allogenic) and may play a role in the inconsistency of production of cartilage and/or bone. In this experiment the initial response to the allogenic material is high and many inflammatory cells have invaded the matrix.

Cartilaginized bone matrix shows a significant potential to establish cartilaginized connections whereas healing between cartilaginous fragments is mostly incomplete [11]. During further growth the lumen of the reconstructed cricoid does not become stenotic. This is in contrast with experimental studies in which hydroxylapatite and autologous cartilage were used for similar cricoid reconstructions [12,13]. These implants did interfere with the normal postnatal enlargement of the cricoidring and actually resulted in a gradually increasing subglottic stenosis.

CONCLUSIONS

The experiments have shown that:

1. The elevated perichondrium of ear cartilage is stimulated by demineralized bone matrix to produce large quantities of mesenchymal cells; these cells invade and colonize the matrix and differentiate into chondroblasts.

2. The cartilaginized matrix exhibits more or less the same dimensions of the previous demineralized bone matrix. In rabbits it appears to be an excellent graft for reconstructing defects in growing cartilage for:
   a. it forms a cartilaginous union with the host cartilage,
   b. it is capable of growth and does not lead to a secundary stenosis during further development up to the adult stage.

Whether such cartilaginization is restricted to young animals or can also be achieved in adult -non growing- animals should be investigated. The most important aspects that need to be addressed relate to the biochemical nature of the perichondrial stimulation and the possibility of the use of induced cartilage in the young child.

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CHAPTER 8

RECONSTRUCTION OF THE GROWING CRICOID WITH A COMPOSITE GRAFT OF DEMINERALIZED BOVINE BONE AND AUTOGENOUS PERICHONDRIUM

ABSTRACT

In growing rabbits the feasibility of a new type of composite graft for the reconstruction of defects in the cricoid ring, is studied. This graft consists of demineralized bovine bone matrix (DBBM) enfolded in a perichondrial flap. The cartilage formed in the DBBM by cells derived from the perichondrium, is demonstrated to provide a valuable substitute for resected parts of the cricoid. A specific feature of this reconstruction is, that it allows further growth and does not result in a secondary stenosis during later development.

INTRODUCTION

Cartilage grafting is a frequently performed procedure when an acquired or congenital subglottic stenosis in children needs surgical correction [1,2]. Successful use of costochondral cartilage grafts has been reported and, for the moment, these seem to be the best available [3]. However, it is not certain whether the growth potential of the cartilage grafts allows a commensurate expansion of the airway with increase in age up to the adult stage.
Infection, resorption and necrosis can complicate the clinical use of cartilage grafts [4]. From animal experiments concerning the viability of the different grafts, contradictory results are reported [3, 5-11]. In general only a minority of the observations were carried out in the growing animal [5,10,12,13]. Exact growth of the various grafts has never been quantified.
Because of the above-mentioned disadvantages of cartilage grafts, continuous interest is attributed to the use of various biomaterials as a substitute in cartilaginous defects. Reconstructions of the cricoid with Proplast and Plastipore [14,15] were not successful. It was advocated that hydroxylapatite can be employed effectively to correct a stenosis in the adult human larynx [16], but earlier experiments have demonstrated that this material induces a subglottic stenosis when used for reconstruction purposes in the growing larynx [17].
Demineralized, dehydrated and sterilized bovine bone matrix (DBBM) has been used in craniofacial surgery as a substitute for bone grafts [18,19,20]. Implanted in skeletal or extra-skeletal sites DBBM is completely replaced by newly formed cartilage and bone [21,22,23]. Recently, it was demonstrated that DBBM, wrapped in a pedicled flap of perichondrium of the aural pinna in young rabbits, is infiltrated by chondroblasts. Within a period of three weeks the cells will form a cartilaginous structure with the same shape and size as the original piece of DBBM [24]. It was suggested that this newly formed cartilage with adherent perichondrium could be used as an appropriate graft.

In this study two types of DBBM-grafts, used for the reconstruction of an incomplete cricoid ring, are compared. The first type of graft is DBBM wrapped in a free transplant of ear perichondrium. The second graft consists of the above-mentioned previously cartilaginized DBBM. As great importance is attached to the treatment of cricoid pathology during growth, the experiments were performed in young rabbits and the effects studied 4-5 months later in the adult stage.

Materials and Methods

Twenty young female New Zealand White rabbits, weighing 400-600 gr were divided in two groups (A and B). Group A animals were operated upon at the age of 4 weeks (one-stage procedure). Group B animals were subjected to a two-stage procedure with surgical interventions at the age of 4 and 7 weeks. Anesthesia was accomplished by administering Ketamin (1 ml/kg) IM and Xylazin-hydrochloride [Rompun] (1 ml/kg) IM.
All animals of both groups were sacrificed at the adult age of 24 weeks.

Group A, one stage procedure (fig. 1)

On the glabrous side of the left aural pinna a skin flap is elevated from the perichondrium by blunt dissection. Next, a perichondrial flap measuring 10x10 mm is prepared. The cricoid is exposed through a cervical midline incision. Then, an anterior part of the cricoid, measuring approximately 3 mm, can be removed without damaging the underlying soft-tissue lining of the airway. The perichondrial flap in the ear, now completely resected, is used to envelope an arc-shaped piece of DBBM which has approximately the same size as the gap created by the resection of the anterior arch of the cricoid. This DBBM-perichondrium graft is interposed between the cut ends of the cricoid. Graft and cartilaginous stumps are connected by absorbable sutures. The muscles, subcutaneous tissue and skin overlying the cricoid are closed in layers; finally, the wound in the skin of the ear is closed.

Group B, two-stage procedure (fig. 1)

Stage I: A pedicled perichondrial flap is created in the left ear, as described above. An arc-shaped piece of DBBM (3 mm in length) is wrapped in the vascularized perichondrium and left in situ. The overlying skin of the ear is closed.

Stage II: Three weeks later, the perichondrium and DBBM graft have been transformed into one solid cartilaginous structure covered by perichondrium [24]. At that time the composite graft can be harvested easily and used for a reconstruction of the cricoid following the procedure as in group A.

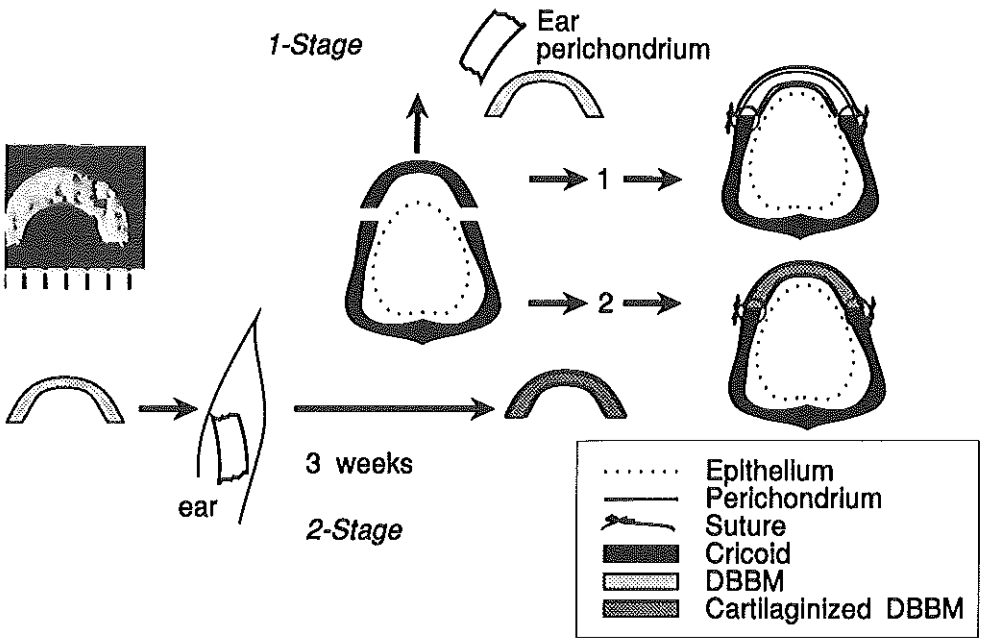

Figure 1

Schematic drawing of the one-stage and the two-stage procedure. Inset shows arc-shaped demineralized bone matrix before implantation. The porosity of this material averages 300-400 μ.

The larynges were fixed in a 10% formaldehyde solution and histologically processed as in previous studies [25]. 5μ thick sections were prepared and stained in a Pas-Alcian blue solution. The histological sections were microscopically assessed.

The subglottic airway lumen was measured by a Zeiss videoplan computer; for measurements, a representative histological section at the level of the reconstructed cricoid ring was chosen. The procedure involves tracing of a magnified (x25) microscopical image of the cricoid ring, the airway lumen and the graft with a sensor light which is detected by the magic eye of the computer. Also the space encircled by the cricoid ring (cricoid lumen) was measured because the subglottic airway lumen in some specimens of this material is partly defined by the presence of a trapped first tracheal ring (figs. 2 and 3).

The length of the DBBM-perichondrium grafts was measured in both groups at the time of implantation. The grafts were measured again in the histological sections of the full-grown larynges. Schematic drawings of the reconstructed cricoids in group A and B were prepared. The drawings were made by tracing a standard magnified light projection of a representative transversal histological section of each cricoid. From previous experiments of this long-term study, microscopical sections of laryngeal specimens of 20 adult rabbits (female, New Zealand White), 24 weeks of age, were available for comparison [5,25].

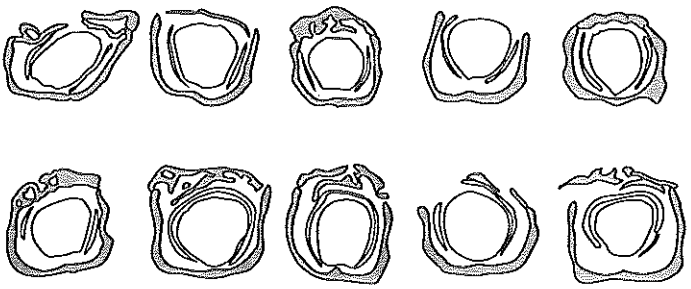

Figure 2
Schematic outline of adult cricoids treated according to a one-stage procedure at young age. In 1 out of 10 specimens no cartilage has emerged from the graft. Note the disorganized aspect of the newly formed cartilage. The graft did not induce a stenosis of the airway.

The demineralized bovine bone matrix (Osteovit®, provided by Braun Gmbh, Melsungen, Germany) is readily available for use.

Results

All animals survived surgery and did well in the postoperative phase.

Macroscopic Observations

After removal of the larynges in 8 out of the 10 group A specimens (one-stage procedure) and all of the group B specimens (two-stage procedure), newly formed cartilage could be clearly perceived macroscopically at the graft recipient site. This cartilage had a dull whitish aspect with an irregular surface in the group A specimens, whilst the grafts in group B seemed to have a more regular aspect.

Microscopic Observations

The cartilage which had emerged out of the graft in the group A specimens showed a significant morphological variability (fig. 2). In most specimens an irregular mantle of cartilage, fibrocartilage as well as hyalin cartilage, surrounded a central core of loose fibrous tissue and clusters of fatty cells (figs. 3 and 4). The amount of newly formed cartilage differed considerably for each specimen; the mantle was never complete and the size of the defects varied to a large extent (fig. 1, fig. 3). One specimen showed no sign of cartilage formation, but a layer of dense fibrous tissue which might be a remnant of the perichondrium. Occasionally a separate perichondrial layer enclosing the cartilaginous parts could be identified in group A. Traces of demineralized bone matrix were sporadically found in some specimens.

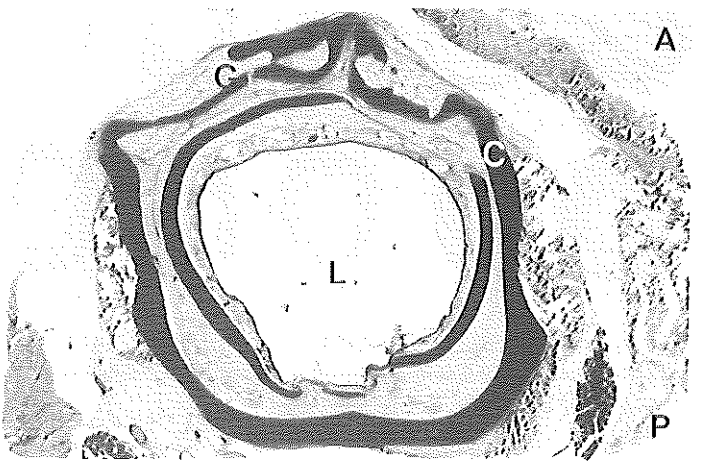

Figure 3

Transverse section of cricoid of adult (24-week-old) animal, subjected to a one-stage procedure at young age (4 weeks). Anteriorly, irregular cartilage has emerged from the DBBM-perichondrium graft. Cartilaginous connections are established. No airway stenosis is induced by the graft. A = Anterior, P = Posterior, C = Cricoid, L = Airway lumen, G = Graft. Magn. 6x, Pas - Alcian blue staining.

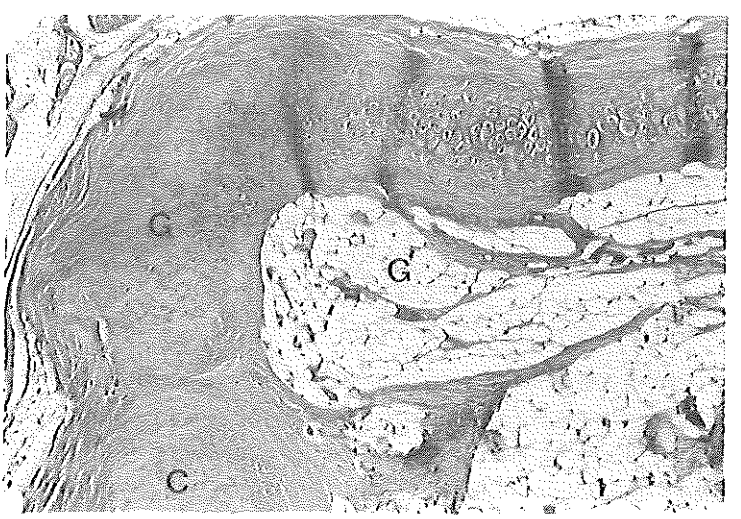

Figure 4

Detailed view of group A specimen. Newly formed hyaline-fibrocartilage, spread at random, is manifest. In between these fields of cartilage, fibrous strings and fatty tissue are present. A real perichondrial layer is absent. C=Cricoid, G=Graft. Magn. 25x, Pas-Alcian Blue staining.

All group B specimens demonstrated an orderly shaped arc of compact cartilage, enveloped in perichondrium (fig.5). The perichondrium consisted of more than one layer; often, the fibres of the perichondrium of the graft were fused with the perichondrial fibres of the

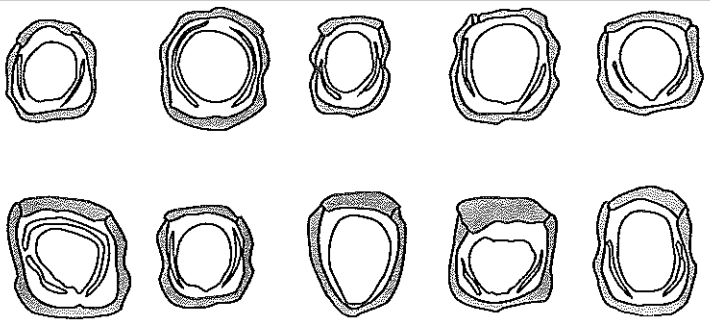

Figure 5

Schematic outline of adult cricoids treated according to a two-stage procedure at young age. In all specimens new cartilage with an orderly aspect is formed. The grafts are tightly fused with the cricoid stumps. No stenosis of the airway has occurred.

cricoid. Histologically, the neocartilage turned out to be partly hyalin and partly fibrous in nature, as are the graft-cricoid connections (figs. 6 and 7). The hyalin cartilage displayed mature chondrocytes, often with a columnar orientation, perpendicular to the perichondrium. Similar to the findings in group A the fibrocartilage contained blood vessels and occasionally some fatty cells.

As far as could be judged from the sections, the demineralized bone matrix had been resorbed in all specimens of group B. No signs of rejection of the graft were observed in the adult stage.

Within the newly formed cartilage, small fragments of bony tissue almost invariably located around a bloodsinus or marrow cavity, were a constant finding. However, the majority of the graft consisted of cartilage.

MEASUREMENTS

The subglottic airway lumen and the cricoid lumen of the group A and B specimens were measured in the transversal histological sections. The mean values are shown in Table 1. The mean surface area of the subglottic lumen in group A specimens (29.12 mm^2) and group B (25.11 mm^2) were significantly different compared to the control group (21,90 mm^2) at $p < 0.01$ and $p < 0.05$, respectively. Statistical analysis (Mann-Whitney test) also demonstrated that the lumen of the cricoid differed significantly in group A (48.90 mm^2) and group B (37.01 mm^2) in comparison with the control group (32.68 mm^2) at $p < 0.01$ for group A and < 0.05 for group B.

The disorderly aspect of the newly formed cartilage in the group A cricoids prevented accurate measurements of the grafts. The grafts in group B averaged 5.7 mm. Since the original length of the implanted graft in vivo was 3.2 mm

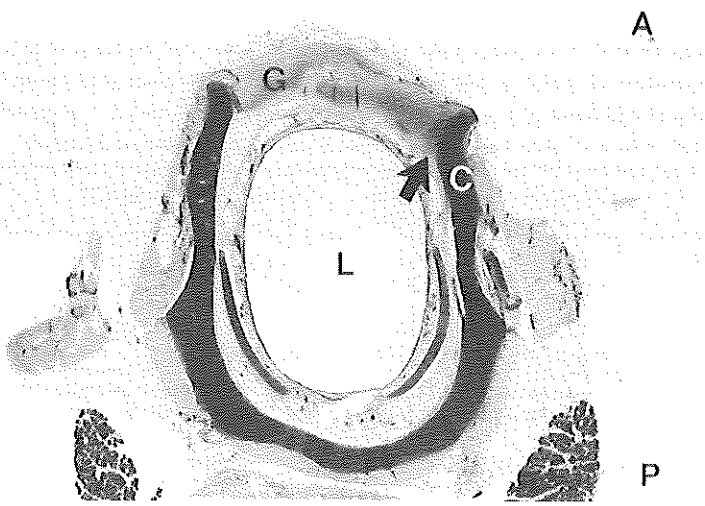

Figure 6

Cricoid of a 24-week-old animal subjected to a two - stage procedure at the age of 4 weeks. Graft (G) is mainly composed of hyalin cartilage and has a regular arc-shaped aspect. Solid connections with cricoid stumps are established (arrow). A = Anterior , P = Posterior , C = Cricoid , L = Airway lumen. Magn. 6x, Pas-Alcian blue staining.

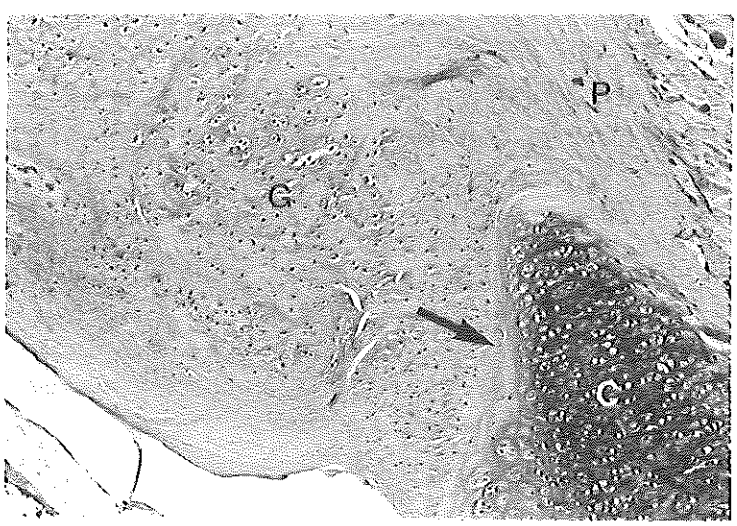

Figure 7

Detailed view of graft-cricoid connection after reconstruction in a two-stage procedure. Arrow indicates transition zone between graft and cricoid. G = mixture of hyalin and fibrous neocartilage in graft, C = Cricoid, P = Perichondrium. Magn. 25x, Pas-Alcian blue staining.

Table 1

Measurements (in mm²) of the mean value of the subglottic lumen and the mean value of the cricoid lumen in specimens of group A (one-stage procedure) and B (two-stage procedure). Data on the subglottic and cricoid lumen of a control series (group 0) are included. Area 1 = mean value subglottic lumen in mm². Area 2 = mean value cricoid lumen in mm². SD = Standard Deviation.

	Group A (n = 10)	Group B (n = 10)	Control (0) (n = 20)	p A/O	p B/O
Subglottic lumen	29.12 SD 4.17	25.11 SD 3.24	21.90 SD 2.90	<0.01	<0.05
Cricoid lumen	48.90 SD 8.19	37.01 SD 5.03	32.68 SD 3.80	<0.01	<0.05

p A/O value for statistical comparison of group A specimen and the control specimens. (Mann-Whitney)
p B/O value for statistical comparison of group B specimen and the control specimens. (Mann-Whitney)

($\pm$ 0.5 mm), a significant ($p < 0.01$) increase in length could be recorded.
The actual length of the graft in vivo could even have been larger, as some shrinkage during the histological processing may have reduced the measured difference.

Discussion

Currently, reconstruction of the cricoid with alloplastic grafts is still not of major importance in clinical practice. This is partially due to the fact that up to now experimental work in this field, has not been very promising [14,15]. Although hydroxylapatite wrapped in perichondrium demonstrated excellent biocompatibility with the host cartilage and good stability, the lack of growth in the alloplastic segment is not compensated by extra growth of the cartilaginous part of the cricoid [17]. Thus, this material can not be used in the reconstruction of the growing larynx.
The results of the study at present suggest that DBBM which is previously cartilaginized by vascularized perichondrium (two-stage procedure) can be satisfactorily applied as a graft in the growing cricoid. The graft remains viable. It provides a valuable substitute for lost parts of the cricoid, forms firm connections with the cut ends and appears capable of growth. Therefore, unlike hydroxylapatite it can be used for reconstruction of the growing cricoid. Whether the graft responds to general growth stimuli or to the expansion of the preserved part of the cricoid ring, is subject to further study.

The fact that the average lumen surface area in the two-stage procedure is smaller than in the one stage procedure can not be explained. Further study will be required to investigate this phenomenon.
With regard to the cartilage formation in the graft, DBBM combined with non-vascularized perichondrium (one-stage procedure) gives far less satisfying results.

Like in experiments with free perichondrial grafts, the amount of cartilage formed and its precise physical shape are unpredictable [26].

During surgery the "fresh" composite graft is markedly softened by body fluid. The stress emanating from the cut ends of the posterior part of the cricoid sutured to the graft [17], can easily stretch and deform the implant at an early stage. In adulthood, the amount of newly formed cartilage is smaller and restricted to a cartilaginous zone, peripheral to the bone matrix. Apparently, the DBBM has irregularly been invaded by chondroblasts originating from the perichondrium. On the other hand colonization by chondroblasts is a striking phenomenon in DBBM enwrapped by a pedicled flap. It seems most likely that the poor circulatory conditions of the free flap prevent a more productive reaction, as observed in the pedicled graft in the ear [24].

The observation of cartilage formation and enchondral ossification confirms data from studies on DBBM, implanted in extra-skeletal sites without any perichondrium contact [21,22,23]. The appearance of bony tissue in the grafts means no disadvantage, as it does not inhibit growth of the cartilaginous cricoid ring.

In the one-stage procedure the core of DBBM just seems to prevent the "collapse" of the perichondrial graft, whereas in the two-staged method DBBM plays a more intriguing role. Essentially, the DBBM is used as a transient model for a cartilaginous graft, to be formed by perichondrium of which the chondrogenic potential is also stimulated by the trauma of elevation [28,29]. Moreover, it has been hypothesized that DBBM itself can promote cartilage formation [21]. The results suggest a near complete biodegradation of DBBM. Consequently, the character of the graft changes from auto-alloplastic [30] to autogenous by virtue of the stimulation of wound healing, chondrifying and regenerative capacities.

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RECONSTRUCTION OF THE ANTERIOR LARYNGEAL WALL WITH A COMPOSITE GRAFT OF DEMINERALIZED BOVINE BONE MATRIX AND AUTOGENOUS PERICHONDRIUM: AN EXPERIMENTAL STUDY IN ADULT RABBITS

ABSTRACT

In this study the feasibility to reconstruct the anterior laryngeal wall in adult rabbits, with a composite graft of demineralized bovine bone matrix and autogenous perichondrium was investigated. It is demonstrated that demineralized bovine bone, which is a porous biomaterial, is completely transformed into autologous cartilage, once it is implanted in a vascularized perichondrial pocket. It appeared to be possible to reconstruct the anterior laryngeal wall in adult rabbits with this newly formed cartilage. As the biomaterial has been transformed into autologous tissue it is entirely incorporated at the recipient site giving rise to solid connections (cartilaginous and/or fibrous) with the surrounding tissues.

INTRODUCTION

The interposition of autologous costal cartilage following a laryngofissure is frequently performed to augment the airway lumen in case of a subglottic stenosis [1,2]. The necessity of a harvesting procedure with the risk of complications and discomfort at the donor site, or the dislocation of the graft due to secondary morphological changes in the cartilage [3,4], are disadvantages to be avoided. In the 1970's perichondrial grafts from ear and rib, which have a chondrogenic potential, were utilized for reconstruction of a defect of the tracheal wall [5,6].

The concept that free perichondrial flaps produce new cartilage had already been demonstrated in experimental animals [7] but the latter studies [5,6] have shown that the neocartilage was very irregular in shape and varied considerably in quantity. Now, 20 years later it is clear that perichondrium alone is not the ultimate solution for closing a laryngeal defect. This has made investigators search for other biomaterials. Earlier experimental and clinical investigations into laryngotracheal reconstruction with an alloplastic material have been reviewed by Bailey and Kosoy [8] and Jacobs [9]. For the reconstruction of the cricoid, hydroxylapatite wrapped in autogenous tissue proved to be the most suitable graft as it is fully incorporated in the cartilaginous cricoid ring [10,11]. However, the use of hydroxylapatite should be restricted to the adult stage. The incapacity of this biomaterial to "grow" interferes with a commensurate expansion of the reconstructed cricoid ring, resulting in a progressive relative stenosis during further growth [11]. Proplast [12] and plastipore [12,13] were demonstrated to be less useful for this purpose because of insufficient incorpor-

ation into the surrounding tissue and hence a lack of stability. Once an alloplastic graft was employed for the reconstruction of the complete anterior subglottic wall (cartilaginous skeleton and soft tissue lining) and thus, exposed to the bacterial flora in the airway lumen, the outcome of the grafting appeared to be disastrous [8,9]. Colonization of the graft by micro-organisms with subsequent extrusion and the formation of abundant granulation tissue in the airway mucosa were the inevitable result. It may be concluded that currently no alloplastic material exists which can withstand this compulsory fate.

A new experimental approach towards the problem of reconstructing the wall of the airway (cartilage and soft tissues) was the use of an alloplastic material in a two stage procedure. Gelfoam with stainless steel mesh [8] or Dacron polyurethane mesh [9] have been utilized after preceding fibrous encapsulation in an extralaryngeal site, to repair laryngotracheal defects. In a similar two-stage-procedure Gady Har-El [14] applied methyl-methacrylate grafts, some of which were additionally wrapped in periost or fascia lata. The results of these experiments suggested that non-degradable biomaterials seem to need an envelope of autogenous tissue to be promising. Yet, a delayed epithelization, the formation of granulation tissue and a collapse of the graft appeared to be awkward complications.

To bypass the problems encountered after reconstruction of the airway with non-degradable biomaterials, we considered the use of biodegradable alloplastic materials like demineralized bovine bone matrix (DBBM). In a previous study in growing rabbits, it was demonstrated that DBBM wrapped in a pedicled flap of autologous ear perichondrium was invaded by mesenchymal cells which differentiate into cartilage. Within a period of three weeks the DBBM is transformed into a cartilaginous structure with sporadic enchondral ossification [15]. In a follow-up study it was shown that the growing cricoid could be reconstructed effectively with a cartilaginized bone matrix [16].

The current study was carried out to answer the following questions:

1. Does cartilaginization of DBBM also occur in adult rabbits when implanted under a pedicled flap of ear perichondrium.
2. If so, is it possible to reconstruct the cricoid of the adult animal using "cartilaginized DBBM" (two-stage-procedure) or fresh DBBM, wrapped in a free perichondrial flap (one-stage-procedure).
3. Can a surgically created window defect of the anterior wall of the subglottic airway in adult rabbits, be successfully closed with a "cartilaginized DBBM"-graft?

Materials and Methods

30 adult female New Zealand white rabbits weighing 3000-4000 gr. were used in this study. These animals were divided in 3 groups: series A (N=10), B (N=10) and C (N=10).

All animals were anesthetized by intramuscular administration of Ketamin 10% (1 ml/kg) IM and Xylazin-hydrochloride 2% [Rompun] (1 ml/kg) IM.

Series A: Two-Stage Reconstruction of the Cricoid and Biopsy of DBBM-Perichondrium Graft.

On the anterior surface of the left aural pinna a skin flap is elevated from the perichondrium, after which a perichondrial flap measuring 10 x 10 mm is prepared. An arc shaped piece of DBBM, measuring 8 x 1.5 x 1.5 mm, is wrapped in the pedicled perichondrial layer. The skin of the ear is closed (stage I). After an interval of three weeks the cricoid is exposed through a cervical midline incision and the anterior one third of the cricoidring is resected, creating a gap of ± 5 mm.

Subsequently, the composite DBBM-perichondrium graft is harvested from the ear, adapted in length to the defect in the cricoid arch and fixed to the cut ends of the cricoid with absorbable sutures. The muscles, subcutaneous tissue and skin overlying the cricoid are closed in layers. Finally, the wound in the skin of the ear is closed (stage II). The superfluous part of each graft (being a biopsy) is used for microscopical assessment.

Series B: One-Stage Reconstruction of the Cricoid.

Essentially, the same methods are used as in series A. A pedicled perichondrial flap measuring 10 x 10 mm is prepared on the anterior cartilage surface of the left pinna. The anterior one-third of the cricoid is resected. An arc shaped piece of DBBM measuring 5 x 1.5 x 1.5 mm is enveloped in the previously prepared and resected free perichondrial flap. The DBBM-perichondrium graft is interposed in the gap (measuring ± 5 mm) between the cut ends of the cricoid and fixed with absorbable sutures.

Series C: two-stage reconstruction of the anterior subglottic wall.

DBBM was allowed to be cartilaginized in a vascularized ear perichondrium pocket as described for series A. In this experiment a larger perichondrial flap is created (20x10 mm) and wrapped around a square piece of DBBM measuring 10 x 10 x 1 mm. After 3 weeks, the cricoid is exposed as described above and the anterior one-third is removed. In addition, a triangular shaped defect of the soft tissue lining reaching from the caudal edge of the thyroid to the first tracheal ring, is created. The DBBM-perichondrium graft is shaped to match the triangular window defect with a (transverse) base of 5 mm and a sagittal length of ± 10 mm and sutured in place to accomplish an airtight closure.

All animals were sacrificed 8 weeks after completion of the one-stage (series B) or two-stage procedures (series A and C). As in previous studies [15-18], the larynges of all series and the biopsies of series A were fixed in a 10% formaldehyde solution and histologically

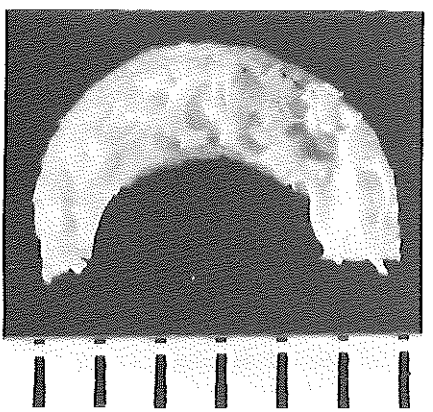

Figure 1a
DBBM. Note porosity of the bone matrix.

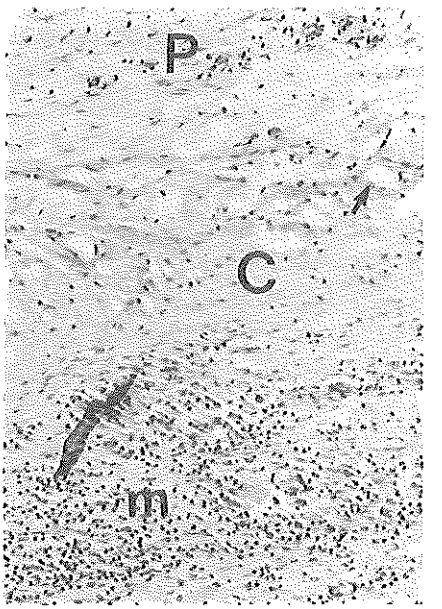

Figure 1b
DBBM, 3 weeks after implantation in a pedicled perichondrial pocket in the ear. Histologically, young cartilage (C) intermingled with mesenchymal cells (M) can be seen. Numerous bloodvessels are observed (arrow). P= perichondrium

processed. Sections of 5μ thick were stained in a Pas-Alcian blue solution.

The demineralized bovine bone matrix (Osteovit®, provided by Braun Gmbh, Melsungen, Germany) is readily available for use (fig.1a).

RESULTS

All animals survived throughout the experimental period without any sign of an airway obstruction.

SERIES A : HISTOLOGICAL ASSESSMENT OF DBBM-PERICHONDRIUM (BIOPSIES).

The perichondrium showed obvious reactive changes: (a) marked thickening and (b) the occurrence of many small mesenchymal cells, especially in the inner layer bordering the bone matrix.

Beneath the perichondrium large areas of chondroblasts and chondrocytes separated by mesenchymal cells and some white blood cells filled up the crevices of the bone matrix; the

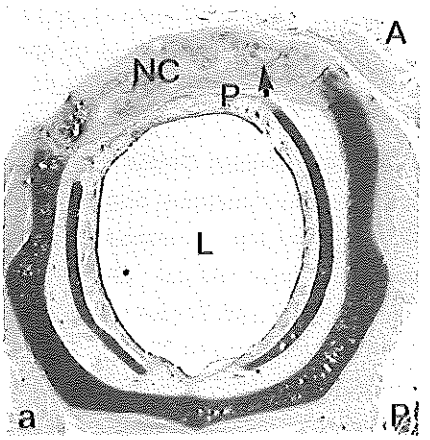

Figure 2a
Cricoid reconstructed with cartilaginized DBBM (two-stage procedure). Graft has an arc shaped configuration. Perichondrial layer (P) surrounds the cartilage. NC = newly formed cartilage in graft with island of bony tissue enclosing marrow cavities (arrow). A = Anterior, P = Posterior, L = Airway lumen. Thick arrow indicates part of first tracheal ring. Magn. x6, Pas-Alc.blue staining.

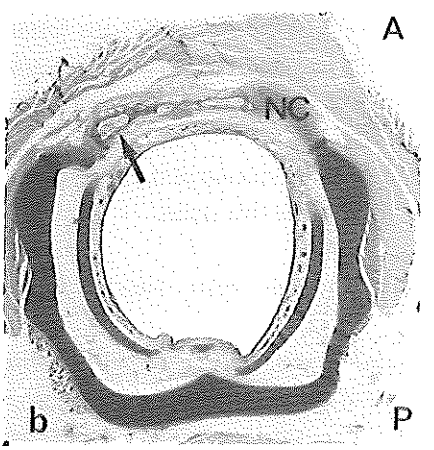

Figure 2b
Transverse histological section of adult cricoid 8 weeks after a one stage reconstruction with a DBBM-perichondrium graft with a slight production of cartilage and bone. Arrow = island of bone which surrounds a marrow cavity. NC = newly formed cartilage.

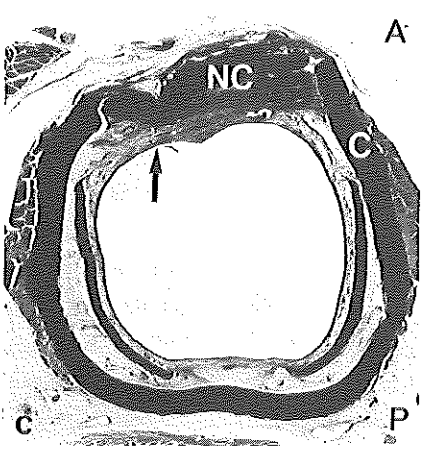

Figure 2c
Cartilaginized DBBM applied for the closure of a triangular defect in anterior subglottic laryngeal wall. C = Cricoid; the graft mainly consists of cartilage (NC) with islands of bony tissue. The inner perichondrial surface of the graft is lined with flat, ciliated epithelium (arrow). The formation of granulation tissue is minimal.

latter had vanished almost completely and only tiny fragments were left. Numerous capillary blood vessels were present in the mesenchymal layers neighbouring small areas of bone, produced by enchondral ossification (fig.1b).

SERIES A : RECONSTRUCTION OF THE CRICOID WITH "CARTILAGINIZED DBBM".

In 9 out of 10 specimen the gap between the cricoid stumps was completely bridged by the slightly curved graft. In the remaining specimen the graft had lost its connection to the cricoid on one side. In all grafts cartilage and bone were found (fig.2a). The cartilage appeared to be of the hyalin and fibrocartilage type intermingled with fibrous strings, fatty tissue and blood vessels. Bony tissue with large marrow cavities occupied 20 to 50% of the total volume of the graft (fig.3). Infrequently, multinuclear giant cells were detected in the vicinity of small remnants of the demineralized bovine bone matrix without significant infiltration of other inflammatory cells.

The graft was covered by a fibrous layer, which seemed to be the remnant of the former thickened perichondrium. The connection between the graft and the cricoid stumps appeared solid and was fibrous, cartilaginous or bony in nature. The cut edges of the cricoid were irregular but did not show signs of necrosis (fig.4).

The rather constant result of the grafting procedure and the large volume of the implant with little variability in size and shape were characteristic features for this series.

SERIES B: ONE-STAGE RECONSTRUCTION OF THE CRICOID WITH FRESH DBBM-PERICHONDRIUM.

In 5 specimens fibrous strings which seemed to originate from the grafted perichondrial layer, and fatty tissue including small islands of cartilage developed. In the other 5 specimens, the graft produced cartilage and bone with a significant variation in amount and shape (fig.2b).

Although the average volume of the latter grafts is smaller than in series A, they demonstrate similar histologic features, including hyalin or fibrous cartilage, bony tissue with marrow cavities, blood vessels and fatty tissue.

SERIES C: TWO-STAGE RECONSTRUCTION OF THE ANTERIOR SUBGLOTTIC WALL WITH "CARTILAGINIZED DBBM".

In all 10 specimens the anterior gap was completely closed and no airway stenosis had developed. In 9 specimens an adequate, solid covering of the defect had been achieved (fig.2c) and the graft consisted of a mixture of hyaline cartilage, fibrocartilage, bone and other tissues covered by perichondrium, as described for series A and B . Except for islands of multinuclear giant cells, no infiltration of the graft by other inflammatory cells had taken place. Besides the attachment of the graft to the cricoid cartilage, a firm fibrous connection

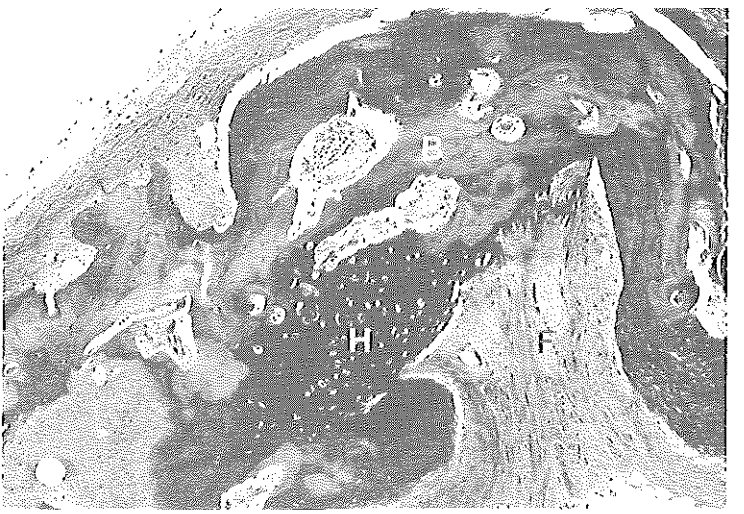

Figure 3

Histological aspect of the graft in a specimen of series A. H = hyalin cartilage, F = fibrocartilage, B = bone with a central marrow cavity. Similar histological features were seen in the series C specimens. Magn. x 25.

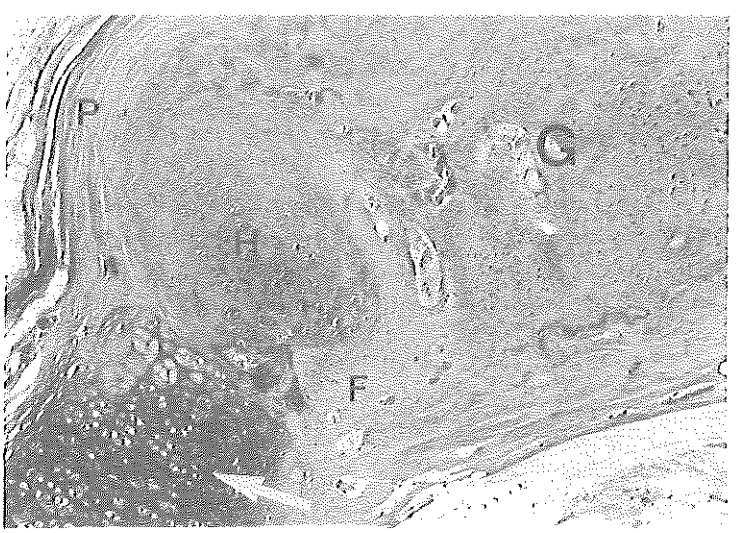

Figure 4

Detailed view of graft - cricoid connection in series A specimen. A solid connection is seen between the cricoid (white arrow) and the graft (G). This connection is partly composed of fibrocartilage (F) and hyalin cartilage (H). Similar histological findings were observed for the specimens in series C. Magn. x 25 . Pas-Alcian blue staining.

had been established with the cricothyroid muscle.

The perichondrium facing the airway lumen, was completely covered by regenerated epithelium. This epithelial layer consisted of flat, cilia bearing cells instead of cylindrical, ciliated cells (fig.5). The formation of granulation tissue appeared to be minimal; in only three specimens insignificant elevations of organized granulation tissue covered by ciliated flat or cubic cells were apparent on the inner surface of the graft. In the tenth specimen the defect was closed by a fibrous membrane lined with flat, ciliated epithelium.

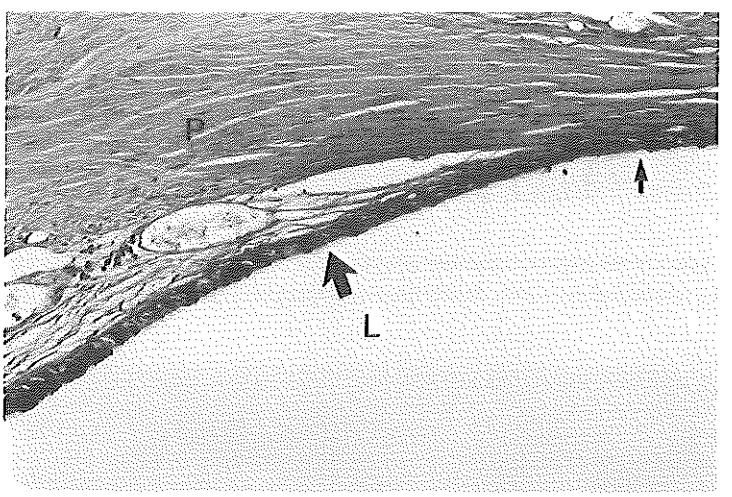

Figure 5
Detailed view of inner surface of cartilaginized DBBM graft used in reconstruction of the anterior subglottic wall (Series C). P = Perichondrial layer, L = Airway lumen. Thin arrow indicates cilia bearing flat epithelium lining the graft. A gradual transition (thick arrow) from the normal ciliated cylindrical epithelium to flat ciliated epithelium is apparent. Magn. x 25, Pas-alcian blue staining.

DISCUSSION

DBBM exerts a remarkable stimulating effect on elevated perichondrium of the ear (series A). The perichondrium responds with thickening and the production of large amounts of mesenchymal cells, which successively leave the perichondrial layer. The reaction of a pedicled perichondrial flap (series A) is much more productive than of a free perichondrial flap combined with DBBM (series B). The differences in reaction between a pedicled and a free perichondrial flap could be explained by the lack of an adequate blood circulation in the latter, with a subsequent crippled metabolism, preventing proliferation of the perichondrial cells. Another possibility is that many cells of the free perichondrial flap become necrotic at

the expense of the productive capacity. Although it must be stressed that the reaction of the perichondrium to DBBM has to be studied in further detail, the first observations in young [15,16] and adult rabbits have not demonstrated obvious age-linked differences during the first 3 weeks. In both age groups the DBBM and perichondrium have been fused to form a structure with a nucleus of mesenchymal cells, chondroblasts and chondrocytes, surrounded by a reactive perichondrium. It appears however that a gradual, age-dependent, divergent histological image develops after reconstruction of the cricoid with a cartilaginized DBBM graft in young and adult animals. In the specimens of the latter group more bony tissue is formed compared to the former group [16].
In 1965 Urist [19] described the formation of bone after extralaryngeal implantation of a demineralized bone matrix and in 1976 Reddi [20] demonstrated an in vitro release of humoral factors from demineralized bone matrix, which could stimulate the formation of cartilage and bone. Our experimental results suggest that these factors are also effective in vivo when the ear perichondrium of young and adult animals is exposed to demineralized bovine bone. The differentiation of mesenchymal cells, the formation of cartilage and bone including the bone marrow, and the eventual fate of DBBM (resolution or incorporation) after implantation in a perichondrial pocket still have to be investigated.

An essential point is that the alloplast is converted into autogenous tissue within 8 weeks. An interval of 8 weeks was chosen, because it is assumed that the process of active woundhealing in cartilaginous structures is terminated at this time [21].
The transformation of the DBBM into autogenous cartilage and bone could explain (a) the excellent viability, the firm connection with the cartilaginous segments of the cricoid and other tissues and (b) the resistance against infection, when exposed to the airway lumen and the absence of granulation tissue. Because of this conversion it is not surprising that DBBM, used in a two-stage-procedure compares favourably to the application of other alloplastic materials as proplast and plastipore [12,13], which are insufficiently incorporated into the surrounding tissues. Non-degradable grafts will remain in the body as a corpus alienum with its potential risks. A propitious exception is hydroxylapatite which was proven to be fully incorporated in young rabbits [11] after reconstruction of the cricoid.
After a histological assessment of the biopsies it became clear that the transplanted graft consists for the larger part of mesenchymal cells and young cartilage; the graft displayed some of the favourable properties of costal cartilage, like viability and rapid epithelization. The drawback, however, is the need for an "incubation period", previous to the use for reconstruction; thus, a two-stage-procedure.
An interesting option for reconstructive surgery could be the paralaryngeal implantation of DBBM which is sandwiched by a (pedicled) perichondrial flap at the inner surface and a muscular layer on the outer aspect. The expectation is that the bone matrix will act as a local

source of "cartilage inducing factors" which could trigger the perichondrium to produce cartilage while a transformation of muscular cells to cartilage and bone, as has been demonstrated by several authors [19,20], is established. Thus, the perichondrium-DBBM-muscle graft is used as the precursor for a future cartilaginous-bony structure which can be applied for laryngotracheal reconstruction.

One final question merits consideration: is it possible that DBBM will induce a chondroma or even worse, a malignancy during the process of rapid cartilage induction? In the opinion of the authors the possibility of these types of uncontrolled growth seems negligible. First, the reaction of the stimulated perichondrium is not unique. Similar phenomena have been described for the elevated perichondrium of the nasal septum adjacent to partly necrotic crushed cartilage [21,22]. The mesenchymal cells produced by the activated perichondrium mature and differentiate into cartilage and other tissues within a period of 8 weeks. In the present study, hardly any immature cells nor signs of stimulated mitotic activity could be found 8 weeks after implantation of "cartilaginized DBBM" in adult rabbits. In previous studies with young rabbits, similar histological features were observed 20 weeks after implantation of "cartilaginized DBBM" [15,16].

CONCLUSIONS

1. Demineralized bovine bone matrix (DBBM) stimulates a pedicled ear perichondrium flap in adult rabbits to produce large amounts of mesenchymal cells. These cells leave the perichondrium, invade the DBBM and for the greater part differentiate into cartilage.
2. This "cartilaginized DBBM" was successfully used to reconstruct defects in the cricoid ring and window defects in the anterior subglottic wall of the airway.
3. Exposed to the airway, without the protection of an overlying mucosa, "cartilaginized DBBM" is resistant against infection, as it is rapidly epithelialized and does not induce the formation of granulation tissue.
4. A free perichondrial flap shows a variable reaction to DBBM.
5. The evolution of grafts of "cartilaginized DBBM" demonstrates age-linked differences.
6. Adding DBBM to a (pedicled) perichondrial graft may be advantageous because the DBBM acts as a source of cartilage inducing factors and as a matrix for cartilage and bone cells.

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CHAPTER 10

SUMMARY AND CONCLUDING REMARKS

The subglottis is the narrowest part of the airway and it is the only part surrounded by a rigid ring, the cricoid cartilage. These two features make the subglottis the most vulnerable region for injury by intubation [1,2]. Apart from that, compression against the cervical spine in case of external trauma, will lead to fracturing of the cricoid ring, displacement of fragments, haematoma and, sometimes, rupturing of the lining mucosa of the airway lumen. Subglottic injuries include a serious risk of causing a subglottic stenosis of the airway, recquiring a tracheostoma and largely impairing the quality of life. Initially, treatment of subglottic stenosis consisted of dilation and intraluminal stenting during several weeks to months [3,4,5]. In the mid-fifties surgical interventions of the laryngeal skeleton were introduced as treatment for laryngeal stenosis [6,7]. Treatment of acquired laryngeal stenosis, especially in children, was recognized as a very difficult task [8,9]. Only a few centres did systematic studies and published their results [10,11,12,13,14,15,16]. Most failures seemed to occur in the group of severe stenosis, in particular in children. Reviewing literature, it was concluded that hardly any information was available on the processes of wound healing after laryngeal injury and the pathogenesis and morphology of the stenosis. The basis for various approaches in treatment was mainly empirical.

The treatment of laryngeal injury and its sequelae has been a point of major interest in the ENT-department of the Sophia Children's Hospital/Erasmus University since the early seventies [17,18]. Consequent documentation of diagnostic data, treatment and long-term follow-up was organized to permit an ongoing evaluation and analysis of treatment results, which will be presented in the thesis of L.J. Hoeve [19]. Parallel to this clinical study, an experimental research project was started to investigate wound healing processes and stenosis formation, and to explore the biological basis for new treatment modalities.
The early animal experiments were described in the thesis of Adriaansen [20,21,22,23,24,25], the consecutive studies in this thesis. Together, they form the largest coherent series of experiments on subglottic stenosis, reported in literature. All experiments have the following features in common:

1. Rabbits have been used as experimental animals (New Zealand White, female).
2. Studies are limited to the subglottic level of the airway and the observations concern morphometry, microscopic anatomy and histology.
3. Housing of the animals, surgical techniques, collection of specimens, histologic processing and morphometry have been performed according to the same protocol.

In Europe no other group has done comparable work on this scale. The department of ENT of the Leuven University recently started experimental work related to tracheal stenosis [26]. Over a longer period, several centres in the USA have been engaged in experimental investigations on laryngeal injury [27,28,29]. Although most of the experiments in these centres initially were focused on dogs, later most colleagues moved on to the rabbit model [30,31,32,33], also advocated by the Rotterdam group. This will favour the future dialogue between the experimental laryngologists involved.

In the previous chapters of this thesis, the results of successive experimental series have been discussed. In this final chapter some of the most relevant new findings and conclusions are presented, in relation to the recent and previous studies from our department.

1. The wall of the subglottic airway in rabbit and man shows a striking similarity in anatomy [24,34]. The epithelial lining of the airway lumen is positioned on a dense layer of elastic fibres. This circular elastic mantle or tunica elastica is fixed to the surrounding framework of laryngeal and tracheal cartilages by a network of elastic bands. Consequently, the subglottic airway wall in rabbit and man consists of three concentric layers from inside to outside:
   a. epithelium and subepithelial elastic fibres (tunica elastica),
   b. an intermediate zone of loose connective tissue, traversed by the elastic bands of the tunica elastica,
   c. the cricoid ring.

   These anatomical similarities favour the rabbit as an experimental animal to study growth and wound healing of the subglottis.

2. Postnatal development of the subglottic larynx in rabbit as well as in man is characterized by:
   a. histologic maturation of the cartilage, especially in the central zone of the cricoid ring,
   b. enlargement in diameter of the cricoid, which at the same time assumes a more ventrodorsal oval shape; the thickness of the cartilage of the cricoid ring hardly increases uptil adulthood,
   c. increase in size of the airway lumen, changing from round to slightly oval [23,24].

The following items (3-7) deal with the effects of endolaryngeal injury (**chapter 6**).

3. The (long-term) effects of subglottic endolaryngeal trauma were studied and described in adult rabbits and compared with the effects in young growing rabbits [20,25]. Determining factors appeared to be:
   I. the depth of the injury;
   II. the age of the experimental animals (young/growing or adult).

4. **Depth of the endolaryngeal injury.** The decisive point is whether or not perichondrium and cartilage of the cricoid ring are involved in the injury. Irrespective of age, superficial lesions, limited to the soft tissue lining, result in a slight or moderate stenosis. The narrowed airway is lined by epithelium, covering a circular mantle of dense collagen tissue, which is separated from the surrounding cricoid ring by a zone of loose connective tissue and fatty tissue. Glands and tunica elastica do not regenerate. Deeper lesions including the soft tissues, the perichondrium at the inner surface of the cricoid ring and the immediately underlying cartilage result in a different histological type of subglottic stenosis. In young as well as adult animals a more or less compact mass of fibrous tissue with cartilage and sometimes bone, is formed between the epithelium and the cricoid ring. This results in a thickened and pathologic intermediate zone, causing a moderate to severe narrowing of the airway. Apparently, the injury of the perichondrium and subperichondrial cartilage initiates the production of a large amount of cells, which differentiate into the components of the stenotic thickening of the airway wall. The decrease in airway lumen is most severe anteriorly (ventrally); there, more scar tissue is formed than posteriorly (dorsally). The epithelium, lining the airway lumen, has numerous villiform projections. Regularly cysts lined by ciliary epithelium were encountered in the intermediate zone.

5. **Age-related differences** in wound healing or stenosis formation were not found after a superficial endolaryngeal trauma. They were, however, observed after a deep endolaryngeal trauma and involved two aspects of the reaction of the injured cartilage not yet mentioned.
   I. the secondary deformation of the cricoid ring;
   II. the loss of regenerative potential of the maturing cartilage.

6. **Secondary deformation of the cricoid ring.** Effects of endolaryngeal injury on the general form of the cricoid ring were for the first time reported by Adriaansen [20] in young animals. Next to thickening of the intermediate zone, this secondary deformation can contribute to narrowing of the airway by inward collapse of certain sectors of the ring. In the adult stage, similar types of injury do not lead to secondary deformities of

the cricoid ring. The form of the cricoid is apparently more stable and rigid in the adult than in young animals [20,21].

7. **Loss of regenerative potential of the cartilage of the cricoid ring.** In the adult stage the wound reaction of the cricoid cartilage is related to the depth of the lesion in the cartilage. If the injury extends into the central zone of mature cartilage, the latter will only show regressive changes; it is evidently not capable of regeneration. On the other hand, perichondrium and a narrow zone of subperichondrial cartilage, would contribute to the wound healing by producing new cells if they are part of the "wound surface". In young animals the central cartilage shows regeneration following a short period of temporary regressive changes. The loss of regenerative potentials of the central cartilage is probably related to the histologic maturation, continuing during postnatal development [20].

Interruption of the cricoid ring can occur as the result of external trauma (fractures) or intently as part of a surgical procedure (splits). The items 8-11 will deal with the effects of single or multiple splits (**chapter 2,3,4**).

8. In literature, different opinions have been put forward as to the therapeutic benefits from splitting the cricoid anteriorly, antero-posteriorly or bilaterally [9,35,36,37]. In this study, the effects of various types of splits were analyzed . The effects can be divided into:
   a. wound healing at the cut edges,
   b. secondary deformation of fragments of the split cricoid,
   c. dislocation of the fragments due to muscle action.

   The first two types are dependent on age, the third is independent of age.

9. **Wound healing of the cut edges in relation to age (chapter 4).**
   In young animals, the cut ends of the cricoid transform into rounded stumps of vital cartilage, covered with perichondrium. In adult animals, the central mature cartilage is apparently not capable of participating in the regeneration processes and shows only regressive changes and finally, necrosis. This coincides with the observations after endolaryngeal injury extending into the central zone of cartilage. The perichondrium and probably a narrow zone of the subperichondrially located layer of cartilage form fibrous tissue, sprouting from the periphery of the cut edges.
   Consequently, the cut edges maintain an irregular surface in the adult stage. End-to-end connection of two fragments of the ring can be realised in young animals by fusion of

the perichondrium on both stumps. In the adult situation, fragments can only be reconnected by fibrous strands.

10. **Secondary deformation of fragments of the split cricoid (chapter 3,4).**
Two or more splits of the cricoid ring in young animals invariably lead to straightening of the separated fragments which loose their normal concave form as sectors of a ring. In adult animals, secondary deformation after splitting of the ring is not or scarcely found.

11. **Dislocation of fragments of the cricoid ring due to muscle action (chapter 5).**
The cricoid (as well as the complete larynx) is supported by a balanced system of laryngeal muscles. After a combined anterior and posterior cricoid split, the balance is disturbed and both halves are rotated outwards. Consequently, the anterior ends are separated by a wide gap, whereas the posterior ends, although often shifted into a side-to-side position, remain in direct contact.

12. **Secondary deformation of the cricoid ring and interlocked stresses in the cricoid cartilage (chapter 2,6).**
Secondary deformation of the cricoid ring was mainly observed following endolaryngeal traumatization and/or splitting in young growing animals and hardly after surgery in the adult stage. It has not been studied yet how much time will elapse between the moment of surgery and the moment these secondary deformities become manifest. However, in a few young animals, sacrificed because of progressive dyspnea, two to three weeks after a deep endolaryngeal injury, secondary deformation of the cricoid ring (irregular collapse) could already be noticed [20]. Inspired by a publication of Fry [38] on deformation of grafted costal cartilage, it is suggested that release of interlocked stresses in the cartilage, results in various injury-specific secondary deformities. Consequently, it should be concluded that release of interlocked stresses plays a minor role in the adult stage than in the young animals. This could be a reflection of the histologic maturation of the cartilage.

The biophysical and chemical basis for interlocked stresses in cartilage deserve further analysis in view of the clinical interpretation of trauma-induced deformities of cartilage. Recently, it was reported that the perichondrium on the exterior surface of the cricoid is much thicker than at the interior side [39]. It could relate to differences in biomechanical conditions on both sides. This feature could also directly contribute to the described secondary deformation due to warping after trauma.

To what extent do the experimental findings previously summarized, help to understand clinical experience in diagnosis and treatment?

13. Papers on the pathology of human specimens with subglottic stenosis are scarce. Hollinger [40] concluded that subglottic stenosis is characterized by a strikingly heterogenous histopathology and suggests a large variability of the processes involved. The observations in the animal experiments (**chapter 6**) may give an explanation for these highly variable pathological findings as the late effects of trauma are apparently determined by the age of the individual at the moment of trauma and the type of injury. Actually, we suggest from the results of the above-mentioned experiments to distinguish the following subtypes of subglottic stenosis, developing after endolaryngeal injury:
    a. soft stenosis with a subepithelial ring of collagen (in young and adult animals after superficial injury),
    b. massive stenosis with deformation of the cricoid ring (deep injury in young individuals),
    c. massive stenosis without deformity of the cricoid ring but with disintegration of the cartilage (deep trauma in adult individuals).

14. A typical form of stenosis, often observed at laryngoscopy in young and adult patients at the subglottic level after a period of intubation, is the (thin and soft) membranous stenosis. Probably the membranous stenosis should be considered the equivalent of the soft stenosis, described in the experiments (type a). Consequently, the membranous stenosis may be the result of a superficial lesion only involving the soft tissue lining of the airway. The more severe types of subglottic stenosis demonstrate at direct laryngoscopy a firm consistency. However, it can not be elucidated at direct laryngoscopy whether the narrowing of the lumen is due to formation of a thick layer of scar tissue including new cartilage (type b) or to the combination of newly formed cartilage and deformation of the cricoid ring (type c).
    Descriptions of the pathology of human laryngeal specimens with severe subglottic stenosis mention dense connective tissue in which no residual normal structures such as glands remain, and ulceration to full-thickness necrosis of the airway wall with sometimes collapse of the cricoid ring [41,42,43,44].
    Biopsies of severe acquired subglottic stenosis in infants have been reported by our research group [45]. Similar findings were registrated as in the massive type of stenosis [type b and c] in the experimental animals. It was stressed that the ectopic cartilage, formed in the thickened intermediate zone, could be misinterpreted as a hamartoma.

A significant inflammatory reaction after a subglottic endolaryngeal trauma was not observed in our specimens collected 8 to 20 weeks after the experimental injury. Therefore a crucial role of perichondritis and chondritis in the pathogenesis of a stenosis, which is suggested by some authors [41,42], could not be confirmed.
In our specimens a local, sometimes irregular thickening of the outer perichondrium and subperichondrial cartilage was a regular finding, which coincides with the observations of Gould et al. in human larynx specimens and was attributed to perichondritis [43,44]. In our opinion it is more a matter of response to the lesion on the inside than an inflammatory reaction of the outside of the cricoid.

Subglottic cysts were regularly found in our specimens, like Couriel and Smith observed in patients with subglottic stenosis [46,47]. Most probably, these cysts have to be considered as inclusion cysts because of their ciliary epithelium lining and not as obstruction cysts of glandular origin as was suggested by the above-mentioned authors.

15. If the subtypes a, b and c of subglottic stenosis could be applied to clinical pathology, it is evident that dilation and stenting could only have success in the membranous stenosis group (type a), where this can not be expected in case of a massive stenosis (type b and c). Those (type b and c) are the cases to be treated by laryngo(tracheo)plasty. It is also clear that laser desobstruction of the airway lumen will be unsuccessful when significant amounts of cartilage/bone are present in the scar tissue. Also laryngo(tracheo)plasty may not be successful in case of the combination of a fibrocartilaginous thickening of the intermediate zone and a collapse of the cricoid ring. Another cause of failure may be the regression or even necrosis of the cartilaginous graft, used to augment the stenotic cricoid ring.

16. Laryngo(tracheo)plasty, including splitting of a (stenotic) cricoid ring followed by interposition of a graft between the cut edges has become a routine procedure for some years [48,49]. Theoretically, such a graft should demonstrate:
    a. no regression, good biocompatibility;
    b. stable connection with the cut edges of the split cricoid;
    c. commensurate growth with the other parts of the laryngeal skeleton to prevent later development of a relative stenosis.

17. Cartilage grafting (even when autologous cricoid cartilage was removed and instantly replaced) bears the risk of regression in various degrees and of a solitary perichondrium-to-perichondrium connection (chapter 2) [50]. Of all biomaterials porous hydroxylapatite was reported as one of the more satisfying for the reconstruction of the laryngotracheal

framework in patients [51] and in experimental animals [22,52,53]. In these studies [22,52] it was demonstrated that a perichondrial envelope promotes the tissue acceptance of the hydroxylapatite graft and prevents extrusion if placed in a subepithelial layer position. However, a cricoid ring with an implant of hydroxylapatite is not capable of normal growth, which results in a relative stenosis in the adult stage [52].

The possibilities of a new biomaterial -porous matrix of demineralized bone- for augmentation of a growing cricoid ring were investigated in a series of experiments, of which some conclusions are summarized in the following paragraph.

18. **Demineralized bovine bone matrix (DBBM)**, is known to induce bone formation when grafted in a subcutaneous position [54,55]. It is used for augmentation of the facial skeleton or reconstruction of the mandibula. Wrapped in a pedicled perichondrial flap, DBBM was demonstrated (**chapter 7**) to "activate" the perichondrium to produce large amounts of new mesenchymal cells, which invade the matrix and differentiate into cartilage. In this way, the DBBM is transformed into a piece of cartilage (stage 1) which has the same form and size as the original DBBM mould. The activating influence of DBBM could be attributed to growth hormones, still present in this mainly collagen matrix. This newly formed cartilage, used as a graft (stage 2) to augment the split cricoid ring, proved to form real fusions with the cartilage of the cut edges and to establish a histological continuity due to a more "powerful" wound healing (**chapter 8 + 9**). Moreover, this graft showed no regression, but actual growth. The cricoid ring reconstructed with this material in young animals developed into a normal or even supernormal dimension. Pirsig [56] used a similar two-stage procedure to reconstruct the cartilaginous **nasal septum** in two young children, with good results during 1 and 2 year follow-up respectively. With these two patients a new modality of cartilage grafting was introduced.

19. The neocartilage formed in the combination of DBBM and a vascularized perichondrial flap proved to be resistant to exposure to the airway lumen. When used to reconstruct the complete anterior wall of the subglottis and upper trachea in adult rabbits, it becomes completely covered (**chapter 9**) by a relatively thin layer of fibrous tissue and epithelium. The graft is firmly attached to the surrounding tissues and in particular the cricoid stumps on both sides; then, no stenosis of the subglottic airway is induced.

20. A free perichondrial flap does not respond sufficiently to the "activating" influence of DBBM, hence no significant numbers of new cells are produced. Thus, direct grafting

of DBBM in a perichondrium envelope (one-step procedure) could sofar not be successfully used for the reconstruction of a defective cricoid ring (**chapter 8**).

Although the experimental observations concern the reaction of a healthy subglottis to various injuries, some statements or speculations with regard to surgical treatment of a pathologic subglottic stenosis, especially in infants and children, may be made:

a. An anterior cricoid split will only be successful in young cricoids, an anterior cricoid split in adults will be futile because of the lack of interlocked stresses in the cartilage
b. One cricoid split anteriorly does not decrease the antero-posterior diameter of the cricoid ring, whereas multiple splits do.
c. Multiple cricoid splits of the anterior cricoid arch result in an altered growth of the cricoid ring.
d. Splitting of the growing cricoid ring should be performed with caution, as these fragments can curl towards the airway lumen (warping and/or collapse), causing obstruction, especially if the cricoid ring is already damaged on the inner side by the endotracheal intubation.
e. Combined anterior and posterior splitting of the cricoid does not result in a collapse as stated by many authors. The insertion of muscles on the cricoid should be respected since they are supposed to rotate the cricoid segments laterally, creating an expansion of the split cricoid ring.
f. After a subglottic endolaryngeal trauma the stenosis is most prominent anteriorly, whereas the posterior side of the subglottic airway is supposed to be more vulnerable to injury by the endotracheal tube.
   Also after controlled circular trauma, the tendency towards stenosis formation is higher on the anterior than on the posterior side of the subglottis. This points to differences in wound reaction between the anterior and posterior wall of the subglottic airway.

The experimental results provided for the first time an explanation for the heterogenous histopathology found in human specimens of subglottic stenosis. They further revealed specific injury-related and age-related phenomena and gave a rational background for thusfar unexplained therapy failures and successes. The observation that DBBM activates adjacent perichondrium to produce large amounts of cartilage-forming cells, stressed the possibility of manipulating wound healing features, even in the adult stage.

The experimental results answered a first set of questions, originating from daily clinical practice but generate at the same time many new questions beyond the domain of morphology like:

a. What are the biomechanical mechanisms underlying the secondary deformation of cartilaginous structures (like the cricoid ring) after local injury?

b. What leads to the loss of regenerative potential of the maturing cartilage?
c. Is it possible to influence wound healing processes and especially to enhance cartilage regeneration by the in situ application of growth factors?

The clinical importance of these questions stress the challenge for further research.

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SAMENVATTING EN CONCLUSIES

De subglottische stenose kan ontstaan na een endolaryngeale beschadiging ten gevolge van langdurige intubatie of als gevolg van een uitwendig trauma van de larynx. Het meest frequent wordt deze stenose van de larynx, die op het niveau van het cricoid (=subglottisch) het meest kwetsbaar is, gezien bij (ex)premature neonaten die langdurig beademd worden/zijn. Factoren die het ontstaan van een subglottische stenose bepalen of beïnvloeden zijn: 1. de duur van de intubatie, 2. de maat van de tube, 3. het aantal reïntubaties, 4. een al dan niet traumatisch verloop van de intubaties, 5. de beweeglijkheid van de patient en dientengevolge de beweeglijkheid van de tube ten opzichte van de luchtweg.
Niet duidelijk was echter welke pathogenetische factoren belangrijk zijn bij het ontstaan van een subglottische stenose. Bovendien is vastgesteld dat patienten met ogenschijnlijk dezelfde stenose niet altijd even goed reageren op dezelfde therapie.

Uit literatuurstudies is gebleken dat de kennis omtrent het ontstaan en de behandeling van een subglottische stenose overwegend empirisch bepaald was.
In 1983 werd door Adriaansen een dierexperimenteel onderzoek naar de reactie van het subglottische gebied op verschillende typen traumata bij jonge konijnen gestart. Voorafgaand hieraan werd eerst een studie van de normale luchtweg van dit jonge proefdier verricht. De belangrijkste conclusies uit dit onderzoek waren:

1. De luchtweg bestaat op het niveau van de subglottis uit drie concentrische buisvormige structuren:
   a. het epitheel met de submucosa
   b. een laag van elastische vezels, tunica elastica, gelegen in de submucosa
   c. het kraakbeen skelet
2. De tunica elastica is als het ware opgehangen aan het kraakbeen skelet door elastische dwarsverbindingen, die op regelmatige afstand van elkaar van centraal naar perifeer verlopen
3. Diverse vormen van anterieure cricoid splits en resecties hebben aangetoond dat het cricoid als geheel niet onmisbaar is voor een patente luchtweg. Waarschijnlijk speelt de tunica elastica hierbij ook een belangrijke rol.
4. Na een endolaryngeaal subglottisch trauma op jonge leeftijd ontstaat, afhankelijk van de diepte van het trauma (alleen weke delen of weke delen + binnenste perichondriumblad en subperichondriaal kraakbeen), resp. een weke delen stenose of een ernstige stenose met kraakbeen nieuwvorming in het littekenweefsel en een malformatie van het cricoid.

De in dit proefschrift beschreven studie is een vervolgstudie die de reactie van de subglottische larynx op verschillende typen traumata inventariseert en het volgende beoogd heeft te onderzoeken:

1. De wondgenezing van de subglottis in relatie tot veroudering van het proefdier en zijn weefsels.
2. Het effect van verschillende typen "cricoid splits" op de morfologie en morfometrie van de cricoidring.
3. Reconstructie van de luchtweg met een DBBM-perichondrium implantaat (DBBM= gedemineraliseerde bovine bot matrix)

De resultaten van de studie van Adriaansen en de in dit proefschrift beschreven experimenten hebben geleid tot een dieper inzicht in de dynamiek en wondgenezing van de subglottische larynx als geheel en het cricoid in het bijzonder. Beide studies tesamen genomen leveren de grootste aaneengesloten reeks waarnemingen bekend in de literatuur.

Circulair endolaryngeaal trauma [hoofdstuk 6]

Na een uitwendige benadering van de luchtweg werd met behulp van een kleine boor een endolaryngeaal subglottisch trauma aangebracht. Na 8 en 20 weken respectievelijk in volwassen en jonge dieren werden de resultaten van deze ingreep histologisch bezien en als volgt samengevat:

a. een oppervlakkige circulaire beschadiging van de subglottis [epitheel en submucosa, zonder beschadiging binnenste perichondriumblad en subperichondriaal kraakbeen van het cricoid] resulteert zowel in jonge (Adriaansen) als in volwassen konijnen (dit proefschrift) tot een matig ernstige stenose voornamelijk opgebouwd uit een band van collagene vezels die juist onder het geregenereerde (metaplastische) epitheel gelegen is. Het is gebleken dat de klieren en tunica elastica na dit trauma niet kunnen regenereren. In deze serie zijn geen leeftijds gebonden aspecten van de wondgenezing waargenomen d.w.z. jonge en oudere specimens vertonen dezelfde afwijkingen.

b. In het geval van een diep trauma van de subglottis [epitheel + submucosa + binnenste perichondriumblad cricoid en subperichondriaal kraakbeen] ontstaat:
   1. een ernstige stenose bestaand uit bindweefsel, ectopisch kraakbeen en bot. Ook hier wordt geen regeneratie van de klieren en tunica elastica aangetroffen. Bovendien worden frequent subepitheliale cysten bekleed met trilhaardragend epitheel waargenomen bij volwassen konijnen, waaruit de conclusie is getrokken dat het insluit(inclusie)cysten betreft in tegenstelling tot de in de literatuur veronderstelde occlusiecysten, die hun oorsprong zouden vinden in de afsluiting van klierafvoergangen.

2. Een deformatie aan de voorzijde van de cricoid ring, zoals gerapporteerd door Adriaansen (jonge konijnen) wordt niet aangetroffen bij volwassen konijnen. Wel valt het onvermogen tot regeneratie van het kraakbeen van het cricoid op, wanneer het trauma tot in de kern van het kraakbeen reikt. Zowel bij jonge als volwassen konijnen leidt een oppervlakkige beschadiging van het binnenste perichondrium en het subperichondriale kraakbeen tot kraakbeen-appositie aan de binnenzijde van de ring en een subsequente onregelmatige verdikking. Geconcludeerd kan worden dat met name **de reactie van het kraakbeen van het cricoid onderhevig is aan verouderings processen van dit weefsel.**

Cricoid split [hoofdstuk 2,3,4,5]
De experimenten met verschillende typen "cricoid splits" dienden ertoe een breder inzicht te verkrijgen in de diverse reacties van de cricoidring na een dergelijk trauma. In de kliniek wordt de cricoid split vaak (als onderdeel) toegepast bij de behandeling van een subglottische stenose.

anterieure cricoid split (ACS) [hoofdstuk 2 en 4]
Na een ACS ontstaat bij volwassen konijnen een minimale opening tussen de uiteinden aan de voorzijde. Dit is in tegenstelling tot de bevindingen bij deze ingreep op jonge leeftijd: een grote verwijding aan de anterieure zijde wordt 20 weken later in het cricoid aangetroffen (Adriaansen).

unilaterale cricoid split (ULCS) [hoofdstuk 3]
Behoudens een opening in de cricoidring wordt een afvlakking van de belendende cricoidsegmenten gezien, en wel zodanig dat in sommige gevallen de ringvorm totaal verloren is gegaan en de segmenten bijna een rechte hoek met elkaar vormen.

bilaterale cricoid split (BLCS) [hoofdstuk 2,3 en 4]
Bij jonge konijnen wordt een verbreding van het achterste cricoidsegment gezien, terwijl het anterieure losse segment eveneens aanzienlijk afvlakt (vergelijk UCLS). De voorachterwaartse (=AP) diameter blijkt hierdoor ook te zijn afgenomen. Bij volwassen konijnen blijkt ook een verbreding van het achterste cricoidsegment op te treden, doch het anterieure segment vertoont geen enkele neiging tot strekking ("stretching") en behoudt zijn normale kromming, waardoor de AP-diameter dezelfde blijft.

Fragmentatie van de cricoidring in 4 delen [hoofstuk 3]
Indien de voorste boog van het cricoid in 2 gelijke delen gespleten wordt, blijkt dat alle fragmenten zich afzonderlijk strekken; hetzelfde geldt ook indien de "arcus" in 4 delen

gespleten wordt. Als bovendien het binnen oppervlak van de arcus beschadigd wordt voor het splijten, blijkt dat de separate fragmenten niet alleen hun curvatuur verloren hebben, maar zelfs doorbuigen naar de andere kant (convex wordt concaaf).

Bij bovenstaande operaties worden de vormveranderingen van het cricoid die opgetreden zijn, toegeschreven aan de "interlocked stresses" die aanwezig zijn in het kraakbeen. Door Gibson en Fry is beschreven dat interlocked stress ontstaat door de waterbindende capaciteit van de mucopolysachariden in de kraakbeen matrix, die een zekere turgor verlenen aan het weefsel. De resultaten van deze onderzoeken kunnen erop wijzen dat de interlocked stresses bij het volwassen cricoid sterk verminderd zijn aangezien de neiging tot "stretching" nagenoeg afwezig is. Bovendien moet geconcludeerd worden dat de centrifugale krachten in de cricoidring groter zijn dan de centripetale krachten en dat heeft mogelijk ook te maken met het feit dat het buitenste perichondriumblad dikker blijkt te zijn dan het binnenste.

Gecombineerde anterieure en posterieure cricoid split (APCS) [hoofdstuk 5]
Deze ingreep, ook wel de operatie volgens Rethi genoemd en veelvuldig toegepast in de kliniek, veroorzaakt bij jonge konijnen een toename van de voorste en achterste dwarse diameter van de cricoidring en dientengevolge een toename van het oppervlak van het cricoid. Bij de operatie treedt anterieur een grote opening op, terwijl de achterste split als een soort scharnier dient van waaruit de beide cricoidhelften als het ware naar lateraal toe hellen. Naast deze helling wordt ook strekking van de cricoid helften waargenomen. Deze observaties hebben geleid tot de hypothese dat:

1. de interlocked stress een rol speelt bij de geconstateerde vormveranderingen, maar mogelijk nog belangrijker dat
2. de m. cricopharyngeus die op het zij-achteroppervlak van de lamina van het cricoid aanhecht, de cricoidhelften lateraalwaarts doet hellen waarbij de achterste split als scharnier fungeert. In de achterste split wordt meestal weer een (kraakbenige) vergroeiing van de segmenten gezien.

Reconstructie van de luchtweg met een DBBM-perichondrium implantaat [hoofdstuk 7,8 en 9]
Gedemineraliseerd bovine bot heeft de eigenschap nieuwvorming van kraakbeen en bot te induceren na een skeletale of extraskeletale implantatie. Van deze eigenschap, voor het eerst beschreven door Senn, Urist en Reddi, is gebruik gemaakt om nieuw kraakbeen te genereren waarmee een reconstructie van het cricoid en de anterieure wand van de subglottische larynx uitgevoerd kan worden.

In een verkennende studie is het DBBM gedurende 1, 2 of 3 weken in een gevasculariseerde perichondrium flap in het oor van het konijn geimplanteerd. De resultaten zijn histologisch onderzocht. Het blijkt dat:

a. na 1 week direct onder het perichondrium een laag van mesenchymale cellen gezien wordt die de botmatrix ingroeit
b. na 2 weken de hele matrix bezet is met mesenchymale cellen die haardsgewijs een differentiatie tot hyalien kraakbeen vertonen
c. na 3 weken de oorspronkelijke botmatrix niet meer alszodanig herkenbaar is doch vrijwel geheel getransformeerd blijkt te zijn tot hyalien kraakbeen; groepsgewijs worden nog wat mesenchymale cellen aangetroffen.

In een vervolg studie werd bij jonge en volwassen konijnen het voorste eenderde deel van het cricoid vervangen door een DBBM-perichondrium graft in een 1 of 2-staps procedure; tevens is bij volwassen konijnen de anterieure subglottische larynx- wand vervangen door een DBBM-perichondrium graft in een 2-staps procedure:

a. 1-staps: een boogvormig stuk DBBM met ongeveer zelfde dimensies als de voorste cricoidboog werd gewikkeld in een lapje vrijgeprepareerd oorkraakbeenperichondrium. Met dit implantaat werd vervolgens de reconstructie van het cricoid uitgevoerd.
b. 2-staps: DBBM werd eerst 3 weken in een oorkraakbeen-perichondrium pocket geimplanteerd om te verkraakbenen. Vervolgens werd deze "graft" gebruikt om het voorste eenderde deel van het cricoid te vervangen.
c. na implantatie in het oor gedurende 3 weken werd de DBBM-perichondrium graft gebruikt om een defect van de totale voorwand van de subglottische larynx te sluiten.

ad a+b: Het is mogelijk gebleken met DBBM in een 2-staps procedure een adequate reconstructie van het cricoid uit te voeren. Niet alleen een goede incorporatie van het nieuwgevormde kraakbeen wordt gezien, met kraakbenige verbindingen naar de cricoidstompen toe, maar ook groei van het implantaat werd aangetoond. Een luchtwegstenose wordt dus door dit implantaat zeker niet opnieuw geïntroduceerd. Indien een 1-staps procedure wordt gevolgd, wordt wel kraakbeen-nieuwvorming gezien, doch de resultaten van deze procedure zijn onvoldoende en niet consistent.

ad c: Een defect in de voorwand van de subglottische larynx blijkt adequaat afgedicht te kunnen worden door implantatie van DBBM in een 2-staps procedure. Zowel met het cricoid als de omringende weke delen worden stevige verbindingen gevormd en het trilhaardragende slijmvlies van de luchtweg regenereert gemakkelijk snel over het implantaat heen.

Concluderend kan gezegd worden dat DBBM in een 2 staps procedure even goede en waarschijnlijk betere resultaten kan geven na reconstructie van de luchtweg vergeleken met kraakbeen implantatie zoals op dit moment gangbaar is.

Op basis van de experimentele waarnemingen in deze studie lijken de volgende additionele stellingen gerechtvaardigd:

a. Een anterior cricoid split zal t.g.v. de interlocked stress alleen nuttig zijn in het jonge cricoid, en t.g.v. het ontbreken van de interlocked stress niet zinvol zijn in het cricoid van de volwassene.
b. Eén mediane anterieure cricoid split (ACS) heeft geen nadelige consequenties voor de voor-achterwaartse diameter van het cricoid. Eén laterale of meerdere anterieure cricoid splits (ULCS-BLCS-fragmentatie) hebben een verminderde voor-achterwaartse diameter van het cricoid tot gevolg.
c. Meerdere anterieure cricoid splits veroorzaken afwijkingen tijdens de groei van het cricoid, maar geen verminderde groei van het cricoid als geheel.
d. Het toepassen van meerdere anterieure cricoid splits bij de behandeling van een subglottische stenose moet met de nodige omzichtigheid geschieden, zeker als het binnenoppervlak van het cricoidkraakbeen beschadigd is: de individuele fragmenten kunnen door een verstoring van de interlocked stress omkrullen en in het slijmvlies van de luchtweg imprimeren. Gevoegd bij het feit dat de voorachterwaartse diameter waarschijnlijk ook vermindert, is een stenose van de luchtweg door deze ingreep geenszins ondenkbaar.
e. Een gecombineerde anterieure en posterieure cricoid split (APCS) veroorzaakt geen collaps van de beide cricoidhelften, waarschijnlijk door de werking van de m. cricopharyngeus die op het zij-achteroppervlak van de cricoidring aanhecht. Deze spier trekt de beide larynxhelften aan de voorzijde uit elkaar.
f. Een circulair endolaryngeaal subglottisch trauma, veroorzaakt een subglottische stenose die het meest uitgesproken is aan de voorzijde van de luchtweg; dit impliceert dat het epitheel en de weke delen aan de achterzijde van de subglottische larynx kennelijk anders op een dergelijk trauma reageren dan het weefsel aan de voorzijde. De rede hiervoor is niet bekend, maar het zou wel kunnen verklaren waarom de incidentie van het ontstaan van een subglottische stenose zo laag is, wanneer het aantal langdurige intubaties en de mate van beschadiging van de luchtweg in aanmerking genomen wordt: meestal is de beschadiging van de luchtweg het meest uitgesproken aan de achterzijde, over de lamina van het cricoid, terwijl juist dit gebied een zekere "resistentie" vertoont tegen stenose vorming.
g. Een belangrijke ontstekingscomponent in de subglottis werd na een endolaryngeaal trauma bij de proefdieren niet gezien; een pathogenetische rol van een chondritis of

perichondritis op de langere duur bij het ontstaan van een subglottische stenose, zoals gemeld in de literatuur, kon niet door ons bevestigd worden.

h. Een fascinerend verschijnsel is het optreden van een locale verdikking van het buitenoppervlak van de cricoidring na een trauma van het binnenoppervlak. Dit wordt ook in menselijke larynxen met een subglottische stenose beschreven.

i. Subglottische cysten werden regelmatig in onze preparaten gezien na een diep endolaryngeaal trauma. De cysten bleken aan de binnenzijde bekleed met trilhaardragend epitheel. Dit impliceert dat:
   - cysten in het litteken weefsel kunnen ontstaan na een endolaryngeaal trauma; de empirische bevindingen zijn hiermee experimenteel bevestigd.
   - cysten waarschijnlijk niet onstaan door afsluiting van de afvoergang van klieren zoals in de literatuur beweerd wordt; waarschijnlijk is er eerder sprake van inclusie van epitheelresten die aanleiding geven tot cyste-vorming.

j. Het is duidelijk dat:
   - alleen een zachte stenose (oppervlakkig trauma jong/oud) behandeld zou kunnen worden met oprekken en stenten
   - een laser-behandeling van een stugge stenose met ectopisch kraakbeen en bot en een aanzienlijke verdikking van het cricoid aan de binnenzijde gedoemd is te mislukken; de histologische observaties in deze studie bieden een verklaring voor de wisselende resultaten die gerapporteerd zijn in de literatuur na laser-behandeling van een subglottische stenose.
   - in geval van een stugge stenose met deformatie van de cricoidring, slechts een uitwendige operatieve benadering van de stenose uitkomst zal kunnen bieden; bovendien is het duidelijk dat bij een ernstige vervorming van de ring (diep trauma in jong cricoid) alleen kraakbeen-implantatie in aansluiting aan een laryngofissuur onvoldoende zal blijken te zijn.

k. Het is bekend dat kraakbeen onderhevig kan zijn aan resorptie en dislocatie; het gebruik van DBBM zou dan een goed alternatief kunnen zijn.

Het is wenselijk en zinvol dat :

- meer menselijke larynxen met een subglottische stenose ter beschikking komen voor onderzoek; alleen dan kan de extrapolatie van de experimentele waarnemingen naar de kliniek getoetst worden
- er prospectieve klinische studies van langdurig geintubeerde patienten verricht worden; de precieze pathogenetische mechanismen van het ontstaan van een subglottische stenose zullen dan hopelijk ontrafeld worden. Met name de invloed van blootliggend, beschadigd kraakbeen op de stenose vorming kan bestudeerd worden

- meer experimentele studies verricht worden naar de exacte rol van de larynx-musculatuur bij het ontstaan van vormveranderingen van het cricoid en dus de luchtweg
- volgende experimentele studies een juist omschreven functie van DBBM voor klinisch gebruik bij de behandeling van luchtwegstenosen definiëren.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 13 Mei 1960 te Paramaribo, Suriname. Na het doorlopen van de Algemene Middelbare School (AMS) te Paramaribo werd in 1978 met de studie Medicijnen gestart aan de Medische Faculteit van de Universiteit van Suriname. In 1982 werd de Medische Faculteit stil gelegd ten gevolge van politieke onrust in Suriname. Omdat de voortzetting van de studie in Suriname niet mogelijk bleek, werd in 1983 de opleiding aan de Erasmus Universiteit te Rotterdam vervolgd. In November 1987 werd de artsenopleiding met succes voltooid. Vanaf Januari 1988 werd als AIO op de afdeling Keel-, Neus- en Oorheelkunde dierexperimenteel onderzoek verricht met als onderwerp: de subglottische stenose. Vanaf 1 April 1992 tot heden is de auteur in opleiding tot Keel-, Neus- en Oorarts op de afdeling K.N.O. van het Dijkzigt Ziekenhuis (hoofd: Prof. Dr. C.D.A. Verwoerd) te Rotterdam.