JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 9 (1998) 249–255

The healing process of palatal tissues after palatal surgery with and without implantation of membranes: an experimental study in dogs

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The aim of this study was to evaluate the wound-healing process clinically and histologically in growing beagle dogs after palatal repair according to von Langenbeck, with and without implantation of membranes of a copolymer of polyhydroxybutyrate 80%–hydroxyvalerate 20% (=PHB-co-HV 80/20). Von Langenbeck's repair was performed in 12 dogs (age 12 wk), while von Langenbeck's repair followed by implantation of PHV-co-HV membranes was carried out in 11 dogs (age 12 wk). Four dogs (age 12 wk) served as unoperated controls. Standardized intra-oral slides of the palate were taken and measurements of the wound surface areas were carried out. Histological sections were prepared at three different ages. The animals were studied until the age of 25 wk. It was found that wound closure after the von Langenbeck's procedure took about 3 wk, while the use of PHB-co-HV membranes after von Langenbeck's repair resulted in complete wound closure after approximately 7 wk after the membranes had sequestered. At the age of 25 wk, the histologic results after the von Langenbeck procedure showed that the entire scar tissue covering the former denuded bony areas was attached to the bone by means of Sharpey's fibres, while after implantation of the membranes only local scar tissue attachment by means of Sharpey's fibres was found. Further research is necessary to develop a membrane which allows wound closure without sequestration of it.

1. Introduction

Animal studies have been carried out to obtain more insight into the effects of surgical repair of lip, alveolus, and/or palate on facial growth. Herfert [1–3] was the first to perform experiments on beagle dogs. He concluded, from a split mouth study on five dogs, that the raising of mucoperiosteal flaps and leaving denuded bone exposed to the oral environment, resulted in maxillary growth impairment. In a series of studies following Herfert's design, Kremenak [4] and Kremenak *et al.* [5, 6] found that unilateral excision of a strip of mucoperiosteum adjacent to the posterior deciduous teeth resulted in maxillofacial growth inhibition.

Wijdeveld *et al.* [7, 8] simulated palatal surgery according to von Langenbeck, in beagle dogs of different ages in a non-body cleft model. Histological evaluation showed that the composition of the palatal scar tissue in the experimental groups remained different from the normal mucoperiosteum, irrespective of the age on which surgery was performed. The scar tissue covering the wound areas adjacent to the posterior teeth lacked large blood vessels and elastic fibres, and showed a mainly transversal orientation of collagenous fibres. The scar tissue was attached to the underlying bone by Sharpey's fibres and the mucoperiosteum was continuous with the periodontal ligament. The combination of these factors in a growing animal might result in a medially directed tensile force on the teeth, resulting in narrowing of the dental arch during or after transition. The authors suggested that prevention of scar tissue attachment to the underlying bone after surgery might lead to a more favourable dentoalveolar development.

In de Braekt *et al.* [9–11] attempted to prevent scar-tissue attachment by separating scar tissue and palatal bone by implantation of biocompatible, biodegradable membranes using the experimental model of Wijdeveld. Premature degradation of the membranes influenced the results negatively but they concluded that with improvement of the material characteristics of the membranes, the development of Sharpey's fibres might be prevented. Leenstra *et al.* [12] studied five different biodegradable materials and they found that membranes made from a copolymer of polyhydroxybutyrate 80%–hydroxyvalerate 20% (=PHB-co-HV 80/20) seemed to be suitable for prevention of development of Sharpey's fibres after von Langenbeck repair in dogs.

The aim of this study was to evaluate the wound-healing process, clinically and histologically, in growing beagle dogs after palatal repair according to

von Langenbeck, with and without implantation of membranes made from a copolymer of polyhydroxybutyrate 80%–hydroxyvalerate 20 (=PHB-co-HV 80/20).

2. Materials and methods

2.1. Animals

The experiments were performed on 27 beagle dogs. In experimental group 1 ($n = 12$; age 12 wk), palatal surgery was performed using the von Langenbeck technique. In experimental group 2 ($n = 11$; age 12 wk), the von Langenbeck technique was used, followed by implantation of PHB-co-HV (80/20) membranes. Four dogs (age 12 wk) served as unoperated controls.

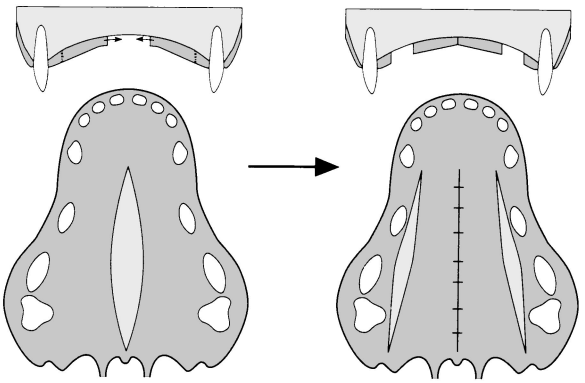

Figure 1 Schematic drawing of the von Langenbeck technique. After surgery, denuded bone is present.

2.2. PHB-co-HV membranes

The PHB-co-HV membranes were prepared at the Department of Chemical Technology, University of Twente, The Netherlands. The material PHB-co-HV (Marlborough Biopolymers, Cleveland, UK) had the following specifications: viscosity average molecular weight (M_v) = 265 kg mol^{-1}, melting temperature (T_m) = 145 °C, glass transition temperature (T_g) = 0 °C, and it had a crystalline structure. The material was dissolved as 5% wt/wt in chloroform (Merck, Darmstadt, Germany). After filtration, membranes were cast on a glass plate in a thickness of 70–80 μm. After evaporation in a nitrogen gas atmosphere for 72 h, the films were vacuum dried at 80 °C for 48 h. The membranes were cut into samples 20 mm × 80 mm. The membranes were treated by radio-frequency glow discharge in order to improve wettability and cell adhesion. A compact electrodeless glow discharge apparatus was used (Harrick PDC-3XG; Harrick, New York, USA). The membranes were placed inside the Pyrex sample tube of the apparatus. When a vacuum of 6.7 Pa was achieved, the system was flushed with argon at a vacuum pressure of 20 Pa. Then, the radio-frequency field was turned on and the membranes were exposed to the glow discharge for 5 min. The glow discharge process was considered to be a sufficient sterilization method [13].

2.3. Surgical procedures

Prior to surgery, the animals were premedicated with 0.5 ml Thalamonal® (fentanyl, 0.05 mg ml^{-1} + droperidol 2.5 mg ml^{-1}; Janssen Pharmaceutica, Beerse, Belgium) and 0.5 ml atropine (atropine sulphate 0.5 mg ml^{-1}). Subsequently, they were anaesthetized with an intravenous injection of 30 mg/kg Narcovet® (sodium pentobarbital 60 mg ml^{-1}; Apharmo, Arnhem, The Netherlands). After intubation, anaesthesia was maintained with Ethrane® (enflurane 15 mg ml^{-1}; Abott, Amstelveen, The Netherlands). The oral mucosa and the dentition were cleaned with chlorhexidine digluconate 1% in water. In addition, approximately 6 ml Xylocaine® (lidocaine hydrochloride 0.4 mg ml^{-1} + adrenaline 0.0125 mg ml^{-1}; Astra Chemical, Rijswijk, The Netherlands) was injected into the palatal mucoperiosteum to avoid excessive bleeding during surgery.

In all experimental animals, a standardized soft-tissue defect was created in the medial region of the palate by incising, elevating and removing an lenticular mucoperiosteal flap. This flap extended distally from the canines to the region of the hard palate distally of the deciduous third molars. The maximum width of the flap was one-third of the transverse distance between the deciduous first molars.

Thereafter relaxation incisions reaching to the bone were made on both sides of the palate adjacent to the posterior teeth. The remaining palatal mucoperiosteum was elevated from the underlying bone with a small raspatory. The major palatine neurovascular bundle was not damaged during the operation. The soft tissue defect was closed in the midline and sutured in one layer with 4-0 Vicryl, leaving two areas of denuded bone adjacent to the dentition (Fig. 1). In experimental group 2, the von Langenbeck procedure was followed by implantation of PHB-co-HV-membranes. The denuded bony areas were measured and the PHB-co-HV membranes were cut to shape with a pair of scissors. Then the membranes were placed on the bone and moved underneath the wound margins for a few millimetres. In each wound, two sutures of 4-0 Vicryl bridging the wound were used to prevent shifting of the membrane. The wound defect was left open to heal by secondary epithelialization (Fig. 2).

All experimental animals were medicated preoperatively with 1 ml Albipen® 15% (ampicillin anhydrate 150 mg ml^{-1}; Mycofarm, de Bilt, The Netherlands) and maintenance doses of 1 ml Albipen® LA (ampicillin anhydrate 100 mg ml^{-1}; Mycofarm, de Bilt, The Netherlands) the second and fourth day postoperatively. All animals received a normal diet after surgery.

2.4. Clinical observations and intraoral slides

Wound healing was clinically observed daily the first 2 wk after surgery and thereafter at each photographic session. Standardized intraoral slides of the palate were taken in all animals at 12 wk of age at the start of

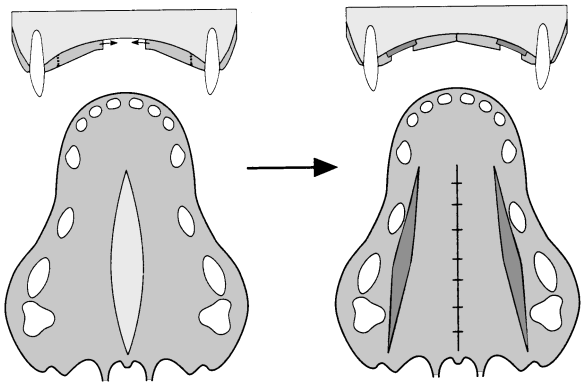

Figure 2 Schematic drawing of the von Langenbeck technique followed by implantation of PHB-co-HV membranes. After surgery, denuded bone is covered with membranes.

the study or prior to surgery and at 1, 2, 3, 5, 7, 10 and 13 wk postoperatively. A Nikon F body (Nikon Corp, Tokyo, Japan) and a Medical Nikon Fixed focus lens (1:5.6, f = 200 mm) with close-up lens and integrated ring flash and Kodak Ektachrome film (200 ASA; Eastman Kodak Rochester, NY) were used. The reproduction ratio was 2:3. A plane intraoral mirror was placed behind the tuberosities of the maxilla at an angle of 45° to the palate. Optimal sharpness was obtained by positioning of the camera and two exposures were made at each occasion. For each photographic session, the animals were premedicated with 0.5 ml Thalamonal® (fentanyl 0.05 mg ml^{-1} + droperidol 2.5 mg ml^{-1}) and 0.5 ml atropine® (atropine sulphate 0.5 mg ml^{-1}). Subsequently, they were anaesthetized with an intravenous injection of 15 mg kg^{-1} Nesdonal® (thiopental sodium 50 mg ml^{-1}). For clinical evaluation of wound healing, the best of each slides on each occasion, was projected and enlarged to trace the contours of the wound areas on paper. Subsequently, they were digitized using an electronic measuring tablet (Hitachi type HDG 11, Hitachi Seiko Ltd, Tokyo, Japan) which had a linear accuracy of 0.2 mm. All tracings and measurements were carried out by one observer. For determination of the measurement error, 20 contours of wound areas were traced and measured twice by the same observer with a time interval of 1 mon.

2.5. Histological processing

For histologic evaluation, animals of each group were killed in pairs. The animals operated according to von Langenbeck were killed at 14, 19 and 25 wk of age. Those operated according to von Langenbeck followed by implantation of PHB-co-HV membranes were killed at 14, 17 and 25 wk of age. The controls were killed at the age of 18 and 27 wk. Prior to perfusion, the animals were brought under general anaesthesia using 30 mg kg^{-1} Narcovet® after which 0.5 mg kg^{-1} Thromboliquine® (Heparin; Organon Teknika, Boxtel, The Netherlands) was administered. After some minutes a lethal dose of Narcovet® was injected intravenously. The thorax of the animal was opened and the vascular system was perfused with physiologic saline via the arch of the aorta, followed by 4% neutral formaldehyde as a fixative. After perfusion the maxillae were dissected and immersed in 4% neutral formaldehyde for another 2 wk. Then, they were sawed into five smaller blocks. Two blocks contained the left and right second premolars and the adjacent lateral palatal areas. Two other blocks contained the third premolars including the palatal areas. The last block contained the midpalatal area between the fourth premolars and the first molars. The blocks were decalcified in 20% formic acid and 5% sodium citrate, dehydrated and embedded in Paraplast® (Monoject Scientific, Athy, Ireland). Serial frontal sections of 7 μm were prepared. For general tissue survey, sections were stained with haematoxylin and eosin. Five sections from each block with a spacing of 175 μm were stained according to Weigert-von Gieson in order to study elastic fibre distribution. All histologic observations were carried out by two independent observers.

2.6. Statistical analysis

The *t*-test was used to compare the wound surface areas between both experimental groups immediately after surgery and at 1, 2, 3, 5 and 7 wk postoperatively.

3. Results

3.1. Clinical observations

Wound healing was completed in the von Langenbeck group and re-epithelialization took about 3 wk. In the experimental group with PHB-co-HV membranes, the first 3 d after implantation, the decrease of the wound surface area was comparable to that of the von Langenbeck group without implantation. Thereafter, the wound surface area increased, until about 1 wk after surgery. Parts of the wound surface had a red inflamed appearance. Complete wound closure took about 7 wk and during that period most membranes were sequestered. Palatal rugae did not develop in the healed areas in either group.

The measurements of the wound surfaces had a duplicate measurement error of 2.0 mm^2. The accuracy of the method was considered to be acceptable. The results of the measurements are presented in Table I.

3.2. Histological observations

3.2.1. Control group

The mucoperiosteum in the control animals was covered with parakeratotic stratified squamous epithelium with many villi protruding into the underlying connective tissue. Just beneath the epithelium, the connective tissue layer consisted mainly of a three-dimensional network of coarse collagen Type I fibres. In the deeper layers, sagitally oriented collagenous fibres were more predominant and elastic fibres were randomly distributed. An expansion tissue was present in those layers with sagitally-oriented large blood

TABLE I Results of *t*-test for comparison of wound surface areas, *x*, of experimental groups

Weeks p.o.	1 Von Langenbeck $n = 12$		2 Von Langenbeck with PHB-co-HV $n = 11$		Significant difference
	X (mm^2)	S.D. (mm^2)	X (mm^2)	S.D. (mm^2)	
0	218.8	32.2	240.0	50.4	ns[a]
1	11.2	8.1	135.6	28.7	2 > 1
2	0.2	0.2	123.0	32.1	2 > 1
3	0.1	0.2	81.5	34.8	2 > 1
5	0.0	0.0	10.8	11.2	2 > 1
7	0.0	0.0	0.0	0.0	ns

[a] ns: $p \geq 0.05$.

vessels along the whole width of the palate. The major palatine artery and branchs of the palatine nerve were found at the lateral aspect close to the bone.

In the youngest animals, at the age of 18 wk the periosteal layer was thick and cell rich. The palatal bone showed trabecular deposition and osteoid formation; active osteoblasts were found on the whole surface and in the mid-palatal suture. Bone deposition was found at the palatal as well as buccal side in the alveolar socket.

At the age of 27 wk the periosteal part of the mucoperiosteum consisted of a thin layer with some resting osteoblasts. Only a few thin collagen Type I fibres connected the fibrous layer of the mucoperiosteum to the bone. The palatal bone was of the lamellar type, no deposition was found. The mucoperiosteum was continuous with the periodontal ligament; cervical periodontal fibres were fanning out into the gingiva and into the deeper layers of the palatal connective tissue.

3.2.2. Von Langenbeck group

In 14 wk old dogs, 2 wk after surgery, the epithelium was almost continuous at the denuded bony areas and consisted of parakeratotic stratified squamous epithelium. The epithelium was thinner and showed fewer protruding villi compared to the epithelium covering the normal mucoperiosteum. The granulation tissue underneath the epithelium was hyperaemic and inflammatory cells were present. Thin collagenous fibres, presumably of type III, were mainly oriented medio-laterally, traversing the former denuded bony areas. No elastic fibres were found in the healing tissue. The formerly mobilized mucoperiosteal flaps located in the medial region of the palate showed a normal appearance. A thin periosteum covered the bone and trabecular bone deposition in the presence of active osteoblasts was found. In the region of the former denuded bony areas, however, apart from osteoblastic bone deposition some local osteoclastic resorption was observed. Simultaneous with the bone deposition, thick collagenous fibres were embedded in the palatal bone as Sharpey's fibres, creating a rigid attachment of the scar tissue to the underlying bone. Collagenous fibres were observed in the cervical part of the periodontal ligament reaching from the cementum into the gingiva and palatal connective tissue. In some instances, osteoclastic bone resorption was found in the cervico-palatal region of the alveolar socket.

At 19 wk of age, the epithelial layer was somewhat thicker compared to earlier stages, especially in the former denuded bony areas but still thinner than in the control animals of comparable age. The medio-laterally oriented collagenous fibres were replaced by thicker fibres, but type III collagen was still predominant. Trabecular bone deposition had decreased but some active osteoblasts were still present in marrow spaces. Sharpey's fibres at the former denuded bony areas were more predominant than in the earlier stages of wound healing and no elastic fibres were present in the mucoperiosteum covering these areas. Cervical periodontal fibres were fanning out into the deeper layers of the mucoperiosteum.

The situation at 25 wk of age closely resembled the stage described previously. The epithelial layer was still somewhat thinner in the operated areas than in the normal mucoperiosteum. The underlying connective tissue contained mainly transverse oriented collagenous fibres and lacked elastic fibres. Rigid attachment of the scar tissue to the palatal bone in the region of the former denuded bony areas by means of Sharpey's fibres was evident. Neither deposition nor resorption of bone was found. Only resting osteoblasts were present. Cervical periodontal fibres were fanning out from the cementum of the premolars to the palatal mucoperiosteum and the scar tissue. In some instances, osteoclasts were observed in the cervico-palatal region of the alveolar socket with corresponding bone resorption.

3.2.3. Von Langenbeck with PHB-co-HV membranes

On examination 2 wk after surgery, the membrane was unimpaired and partly covered with mucoperiosteum (Fig. 3). The bone was of a trabecular type and showed deposition although in some areas resorption was found. A cellular capsule, mainly consisting of neutrophilic granulocytes and macrophages, surrounded the membrane except for the uncovered area (Fig. 4). The mucoperiosteum partially covering the membrane was hyperaemic and showed a cellular

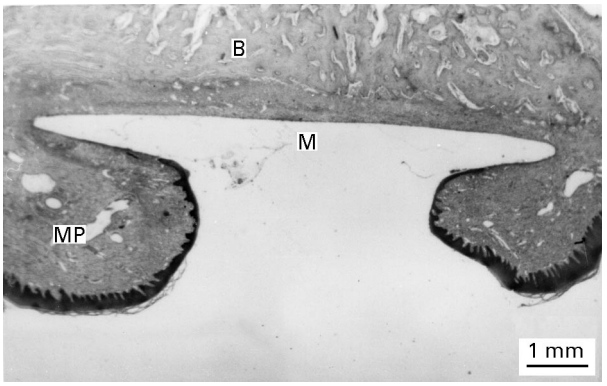

Figure 3 Two weeks after surgery (age 14 wk), the membrane was unimpaired and partly covered with mucoperiosteum. B = bone, M = membrane, MP = mucoperiosteum. H and E staining.

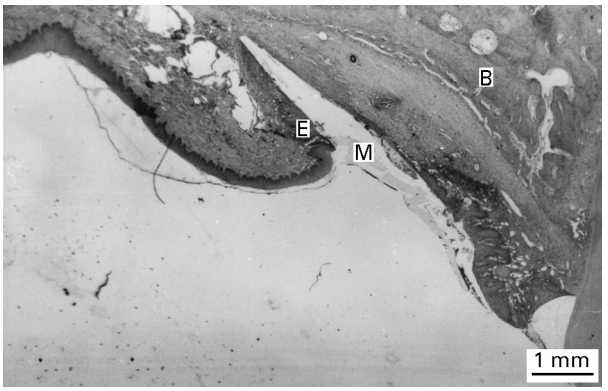

Figure 5 Five weeks after surgery (age 17 wk), the membrane was uncovered at the lateral side while the medial side was still covered with mucoperiosteum. B = bone, E = epithelium, M = membrane, H and E staining.

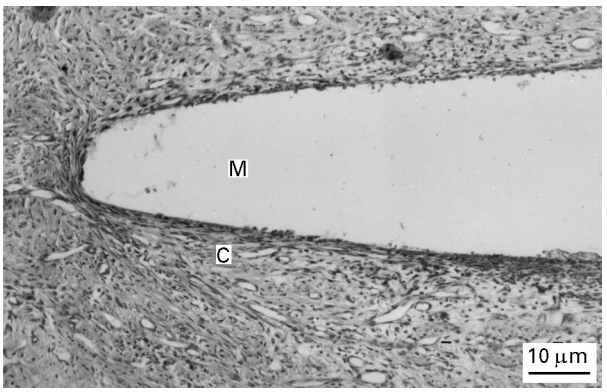

Figure 4. Detail of Fig. 3, showing a cellular capsule, mainly consisting of neutrophilic granulocytes and macrophages, surrounded the membrane except for the uncovered area. C = capsule, M = membrane, H and E staining.

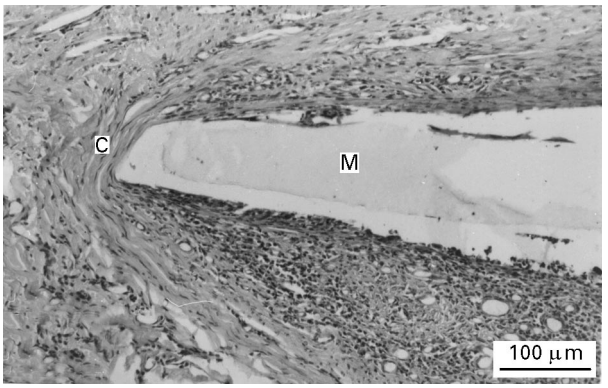

Figure 6 Detail of Fig. 5, the membrane was partly bounded by epithelium and by a fibrous capsule containing lymphocytes. C = capsule, M = membrane, H and E staining.

infiltrate. Ingrowth of epithelium from the edges of the mucoperiosteum was found at the oral side of the membrane.

At 17 wk of age the membrane was uncovered at the lateral side of the palate while the medial side of the membrane was still covered with mucoperiosteum. The membrane was partly bounded by epithelium and by a fibrous capsule containing lymphocytes (Figs 5, 6). Exudate was found at the bony side of the membrane and gingivitis had developed where the membrane was in contact with the gingiva. The bone consisted of lamellar depository bone and no osteoclastic activity was found.

At 25 wk of age, no membrane could be found (Fig. 7). The epithelial layer was still somewhat thinner in the operated areas than in the normal mucoperiosteum. The connective tissue layer consisted mainly of a three-dimensional network. Rigid attachment of the scar tissue to the palatal bone by means of Sharpey's fibres was found in some regions of the former denuded bony areas. The palatal bone was of the lamellar type, and neither deposition nor resorption was found. Only resting osteoblasts were present.

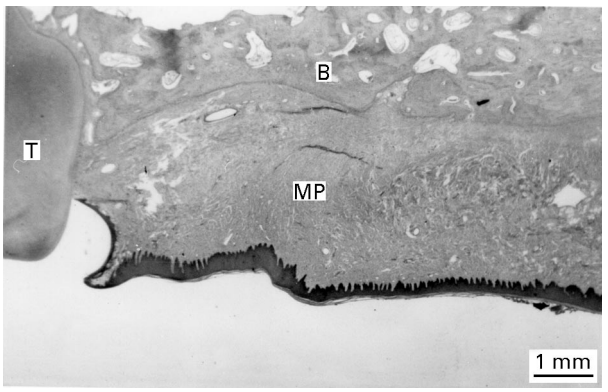

Figure 7 Thirteen weeks after surgery (age 25 wk), no membrane could be found. B = bone, MP = mucoperiosteum, T = tooth. H and E staining.

4. Discussion

The healing process in dogs after palatal repair according to von Langenbeck with and without implantation of membranes of PHB-co-HV during growth was studied clinically and histologically and compared with controls. The goal of the implantation of membranes was to create a temporary barrier, preventing the development of scar tissue attachment to the underlying bone by means of Sharpey's fibres and so to overcome one of the adverse side effects of surgery and to favour dento-alveolar development.

Wound healing was completed uneventfully in the von Langenbeck group and re-epithelialization took about 3 wk. Histologically the wound healing was comparable to the findings of Wijdeveld *et al.* [8] and Densho [14], the latter reporting scar tissue attachment after healing of wounds in which mucoperiosteum was removed and consequently palatal bone was exposed. The formation of bundle bone in the relaxing incision areas after von Langenbeck's repair, is the result of the wound healing in areas of denuded bone. Bundle bone can be characterized as immature bone and often appears in postnatal life in places where bone repair takes place [15].

In the von Langenbeck group followed by implantation of PHB-co-HV membranes, the first 3 d after surgery, the decrease of the wound surface area was comparable to that of the von Langenbeck group without implantation. Thereafter, the wound surface area increased until about 1 wk after surgery. Wound closure needed about 7 wk and, during that period, parts of the wound had an inflamed appearance. The membranes were sequestered at some moment in this period. The reasons for re-opening of the wounds might be weak adhesion of the soft tissues to the membrane surface compared to the forces generated by wound contraction. This in spite of radio-frequency glow discharge treatment in order to modify the surface characteristics of the membranes in favour of cellular behaviour [16]. Prior to the use of PHB-co-HV membranes in growing dogs, the effect of this kind of surface treatment on attachment, spreading and growth of fibroblasts was tested in an *in vitro* study. It was found that treatment by radio-frequency glow discharge of the membranes enhanced the adhesion of fibroblasts significantly. In the present animal study, however, the effect of radio-frequency glow discharge might be disturbed by contamination of the surface directly after implantation [13]. This can have influenced the adhesion of fibroblasts to the membrane surface negatively. Also geometric surface properties of the implant material play a role in cell spreading and locomotion [17]. Unfavourable geometric surface properties (smooth surface) of the used membrane might also be a reason for weak cell–membrane adhesion. The partial exposure of the membrane surface to the oral cavity and bio-incompatibility might be reasons of inflammation of the surrounding tissues finally resulting in sequestration. However, in an earlier study by Leenstra *et al.* [12], in which samples of 4 mm × 8 mm of PHB-co-HV membrane were implanted submucoperiosteal in dogs, the membranes remained *in situ* 12 wk and induced very little tissue response. In this study, the exposed membrane area was relatively small (defect; 13 mm^2) compared to exposed area in the present study (defect; 100 mm^2). Considering these results, it is assumed that overgrowth of the exposed area with fibroblasts is not possible when the area exceeds a critical dimension. On the other hand, In de Braekt *et al.* [9, 11] found wound closure without sequestration after implantation of porous poly(*L*-lactic) acid (=P*L*LA) membranes ($M_v = 220\,kg\,mol^{-1}$, thickness 110 μm, porosity range 0.5–1.0 μm) in the palate of dogs, although the healing was also retarded. Besides different surface characteristics, early fracture of the membranes into several small parts was reported in the studies of In de Braekt *et al.* [9, 11], which might have facilitated wound closure.

The histologic findings at the age of 25 wk after implantation in the present study showed rigid attachment of the scar tissue to the palatal bone by means of Sharpey's fibres in some regions of the former denuded bony areas. The connective tissue layer consisted mainly of a three-dimensional network. The palatal bone was of the lamellar type and neither deposition nor resorption was found. These results are supported by the study of In de Braekt *et al.* [11] in which, in spite of premature disintegration of P*L*LA membranes, scar-tissue attachment to the underlying bone was prevented at sites where membrane particles persisted. Apparently, the time needed to prevent scar-tissue attachment, at least to some extent, by implantation of PHB-co-HV membranes is less than 7 wk because the membranes were sequestered in this period. In spite of the loss of the membranes, the histologic results 13 wk after implantation showed only local scar-tissue attachment by means of Sharpey's fibres while after the von Langenbeck procedure, the entire scar tissue covering the former denuded bony areas was attached by means of Sharpey's fibres. Further research is necessary to develop a membrane which allows wound closure, while the membrane itself remains *in situ*.

Acknowledgements

The authors thank Professor J. Feijen, P. J. Dijkstra, J. G. A. Terlingen, and P. van de Witte (Department of Chemical Technology, University of Twente, The Netherlands) for manufacturing the membranes and for their intellectual support.

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Received 8 July
and accepted 1 September 1997