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Local tolerance and efficiency of two prototype collagen matrices to increase the width of keratinized tissue

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Abstract

Aim: To evaluate the local tolerance and efficiency of two experimental collagen matrices to increase the width of keratinized tissue.

Methods: In 12 pigs, two apically positioned flaps were prepared on both sides of the mandible. The denuded defect areas were randomly covered with one of two experimental porcine-derived collagen matrices (M1; M2). The other defect area was left untreated (control). At 1 and 6 months, clinical measurements for the width and thickness of the keratinized tissue were recorded. At 6 months, all animals were sacrificed. Descriptive and semi-quantitative histologic analyses were performed. For statistical analysis, the Kruskal–Wallis test and the Mac Nemar test were applied.

Results: The collagen matrices integrated well into the surrounding tissue without any signs of inflammation. The thickness and width of the keratinized tissue increased significantly over 6 months in all the groups, resulting in slightly more favourable results for M1 (compared with M2) with respect to the thickness and for M2 (compared with M1) with respect to the width of keratinized tissue. No statistically significant differences were observed for any of the evaluated clinical and histologic parameters among the three treatment modalities.

Conclusions: Within the limits of this animal study, the prototype collagen matrices can be used safely to increase the width of keratinized tissue.

Key words: animal model; apically positioned flap; collagen matrix; keratinized tissue; soft tissue augmentation

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Grafting procedures using autogenous soft tissue transplants are well established in daily clinical practice (Seibert 1983, Orsini et al. 2004, Thoma et al. 2009). However, surgical difficulties, lack of colour match, and an increased patient morbidity represent major disadvantages (Farnoush 1978, McGuire & Nunn 2005, Griffin et al. 2006, Soileau & Brannon 2006, McGuire et al. 2008). To overcome these problems encountered with autogenous tissue, various techniques and materials predominantly of allogenic origin have been developed. Among the first products introduced in dentistry were acellular dermal matrix grafts (ADMG). ADMG was originally developed for covering full-thickness burn wounds (Wainwright 1995) and were later used in dentistry to increase the width of keratinized tissue and for root coverage procedures (Wei et al. 2000, Aichelmann-Reidy et al. 2001, Harris 2003). Even though ADMG is still in use, the results from clinical studies are not convincing due to the high shrinkage rate and the histologic findings demonstrating a tissue, which is substantially different from the oral mucosa when ADMG is used uncovered to increase the width of keratinized tissue (Wei et al. 2000, Wei et al. 2002). However, similar histologic outcomes have been reported for ADMG and autogenous connective tissue grafts placed underneath a coronally advanced

Conflict of interest and source of funding statement

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flap for recession coverage (Nunez et al. 2009). Recently, a cellular component has been added to dermal replacement grafts. The results from various studies suggest that these tissue-engineered grafts may be more favourable in terms of clinical outcomes, but are still associated with a difficult clinical handling (McGuire & Nunn 2005, McGuire et al. 2008).

In contrast to tissue-engineered products derived from human skin, collagen devices from xenogenic origin have been used successfully in dentistry since years. Resorbable collagen membranes are used as barrier membranes for guided tissue and bone regeneration (GTR and GBR) (for a review, see Hammerle & Jung 2003). It has been demonstrated that resorbable collagen membranes fulfil a number of criteria that are a prerequisite for successful use in GBR and GTR procedures: (i) biocompatibility, (ii) tissue integration, (iii) nutrient transfer, (iv) cell occlusiveness, (v) ability to maintain space, and (vi) ease of clinical handling (Hardwick et al. 1994).

The most commonly used resorbable membrane is a collagenous bilayered membrane of non-cross-linked porcine type I and III collagen fibres (Hammerle & Karring 1998, Hammerle & Lang 2001, Friedmann et al. 2002, Oh et al. 2003). A recent study demonstrated a high degree of anastomosis of the vasculature in the regenerated tissue that was induced by the collagen membrane after subcutaneous implantation in rats (Schwarz et al. 2006).

Recently, a prototype collagen matrix with characteristics similar to the most commonly used resorbable collagen membrane was developed with an additional indication to further influence the healing cascade and reduce scar retraction in periodontal defects, and as a replacement for autogenous tissue to increase the width of keratinized tissue. Results from a randomized-controlled clinical trial demonstrated that this newly developed collagen matrix was as effective and predictable as the gold standard, the connective tissue graft, for attaining a band of keratinized tissue (Sanz et al. 2009). However, local tolerance and tissue integration on a histologic level have not been assessed as yet.

Therefore, the aim of the present animal study was to evaluate and compare the local tolerance and performance of two experimental collagen matrices to increase the width of the keratinized tissue by macroscopic and microscopic analyses.

Materials and Methods

Animals

The present study was designed as a randomized-controlled experimental study using 12 Seghers Hybrid pigs. At the beginning, the animals were more than 2 years of age, weighed between 50 and 61 kg, and were kept in a purpose-designed room for experimental animals. The study was conducted in accordance with the requirements of the FDA ''Good Laboratory Practice'' (GLP) Regulations and the ''Bonnes Pratiques de Laboratoire (BPL) described in the ''*Journal Officiel du Ministère Français de l'Emploi et de la Solidarite*''. The protocol was submitted to and accepted by the internal Biomatech NAMSA ethical committee.

Study materials

Prototype collagen matrix 1 (M1) and 2 (M2) (Geistlich Pharma AG, Wolhusen, Switzerland) were prepared from native porcine collagen without additional chemical crosslinking. Both matrices were mainly composed of collagen I and III. M1 was an earlier prototype prepared from the same porcine collagen source as a final product (Mucograft®, Geistlich Pharma AG). M2 was characterized by physical, mechanical, and biological properties very similar to those of the final product (Mucograft®, Geistlich Pharma AG), differing only in the porcine collagen source used.

Anaesthesia and medication

On the day of surgery, each animal was anaesthetized according to a standard procedure: tranquillization by atropine (Atropine®, Aguettant, Lyon, France, intramuscular), induction by tiletamine-zolazepam (Zoletil®100, Virbac, Carros, France, intramuscular), and then thiopenthal sodique (NesdonalND, Merial, Lyon, France, intravenous), followed by inhalation of an O_2–N_2O isoflurane (1–4%) mixture. A pre-operative injection of a local anaesthetic (Lignospan® special, Septodont, Saint-Maur-des-fossés, France) was administered. Prophylactic antibiotic treatment with spiramycine and metronidazole (Stomorgyl®, Merial) was initiated on the day of surgery and was continued for 10 days thereafter. Each animal received carprofene (Rimadyl®, Pfizer, Paris, France, per os) for analgesia for 3 days.

Surgery

Before the surgery, measurements of the width and thickness of the keratinized tissue were performed using a periodontal probe. In order to reproducibly measure the width of the tissue and to further identify the surgical site, four tattoo points were placed coronally, apically, mesially, and distally of each defect site. In addition, based on impressions on each side of the animals, individual acrylic stents were used to perform five repeatable and standardized measurements of the tissue thickness (Fig. 1). The surgical area was disinfected (Vetedine®, Vetoquinol, Lure, France). In each animal, two standardized gingival defects (1 cm vertical × 3 cm horizontal distance) were created bilaterally on the buccal side of the mandible. Two releasing incisions were made into the vestibular mucosa defining the lateral borders of the defect (Fig. 2a). To elevate the flap, a horizontal incision was made in the keratinized gingiva 2 mm coronal to the mucogingival line. A split-thickness flap was elevated, creating a standardized soft tissue defect (1 cm vertical, 3 cm horizontal distance) (Fig. 2b). The horizontal border of the mucosal flap was sutured to the underlying periosteum by interrupted single sutures (Fig. 2c). The denuded defect areas in each hemi-mandible were randomly covered with one of the two experimental porcine-derived collagen matrices (M1, M2) of the exact same size as the defect (Fig. 2d); the other defect was left untreated (control). The apical margin of the matrix was fixed to the periosteum by interrupted single sutures. Subsequently, the matrix was securely stabilized and held in place

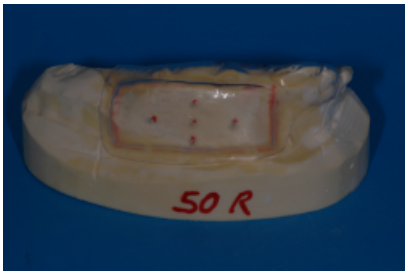

Fig. 1. Individualized stent on a cast model used for repeated measurements of soft tissue thickness.

by cross horizontal mattress sutures. The sutures were removed 14 days following the surgery. The animals were fed a soft diet for the remainder of the study.

Clinical observations and measurements

At 1 month, all animals were anaesthetized (Zoletil100, Virbac, France, and atropine, Aguettant). Measurements of the thickness and width of the keratinized gingiva were performed using a periodontal probe and the individual acrylic stent. The defect sites were checked for any sign of inflammation or local intolerance. Photographs from the defect sites were taken (Fig. 2e). The reepithelization of the defect sites was evaluated on the photographs by digital planimetry using a grid with 289 intersections and a 0.5 mm distance between them. The reepithelization is expressed in percentage of the entire grafted area. In addition, nine biopsies (three of each treatment group) were harvested in three animals.

At 6 months, the same macroscopic observations and measurements were performed as at 1 month.

Colour match

For colorimetric analysis, the clinical photographs taken at baseline (before surgery), after surgery, and at 1 and 6 months were digitized. The images were then assessed (Adobe Photoshop, version 7.0.1) and analysed according to standard colorimetric parameters (Commission Internationale de l'Eclairage [CIE] Lab; L = lightness, a = chroma along the red–green axis, and b = chroma along the yellow–blue axis). Two areas, one from the defect site and one from the adjacent keratinized tissue, were chosen for comparison (Fig. 2f). The colorimetric difference between the two areas (ΔE) was calculated according to the following equation: $\Delta E = [(L_{\text{Graft}} - L_{\text{Adjacent tissue}})2 + (a_{\text{Graft}} - a_{\text{Adjacent tissue}})2 + (b_{\text{Graft}} - b_{\text{Adjacent tissue}})2]1/2$ (Jung et al. 2004).

Sacrifice

Six months following surgery, the animals were euthanized by a lethal dose injection of Dolethal® (sodium pentobarbital, Vetoquinol). The soft tissues were resected together with the underlying bone and adjacent intact gingiva. The coronal border of each biopsy was identified by means of a suture thread. Each sample was identified and fixed in 10% buffered formalin.

Histologic processing and analyses

After fixation, each sample was decalcified (NEW DECALE, HistoLab, Sweden), dehydrated in alcohol solutions of increasing concentration, cleared in isoparaffin H, and embedded in paraffin. Embedded samples were cut at 5 µm using a microtome (MICROM®, Francheville, France) into three blocks (anterior, medial, and posterior, according to the corono-apical axis). One section per block was prepared and stained with Masson's Trichrome.

All histological sections were evaluated using a Nikon microscope (ECLIPSE E600, Nikon, Egg, Switzerland). The following parameters were assessed: inflammation and local tolerance (fibrin, necrosis, tissue degeneration, polymorphonuclear (PMN) cells, eosinophilic PMN cells, lymphocytes, plasma cells, macrophages and giant cells, tissue regeneration, new vessel formation, membrane degradation, remnants, soft tissue maturation).

Statistical analysis

Statistical analyses were performed to compare the two test matrices and to compare each matrix with the control site at baseline, and at 1 and 6 months (SAS® software, SAS institute, Cary, NC, USA). Current statistics (mean, standard deviation, minimum, and maximum) were used to describe the quantitative parameter. At baseline, comparability of the groups was assessed using the Student test or the Wilcoxon test according to the distribution of the parameter. The comparison of groups was performed using the methodology of analysis of variance. The validity conditions of all analyses of variance were verified. Current statistics (frequency and percentage) were used to describe the ordinal parameter. The comparison of groups was performed by site using the Kruskal–Wallis

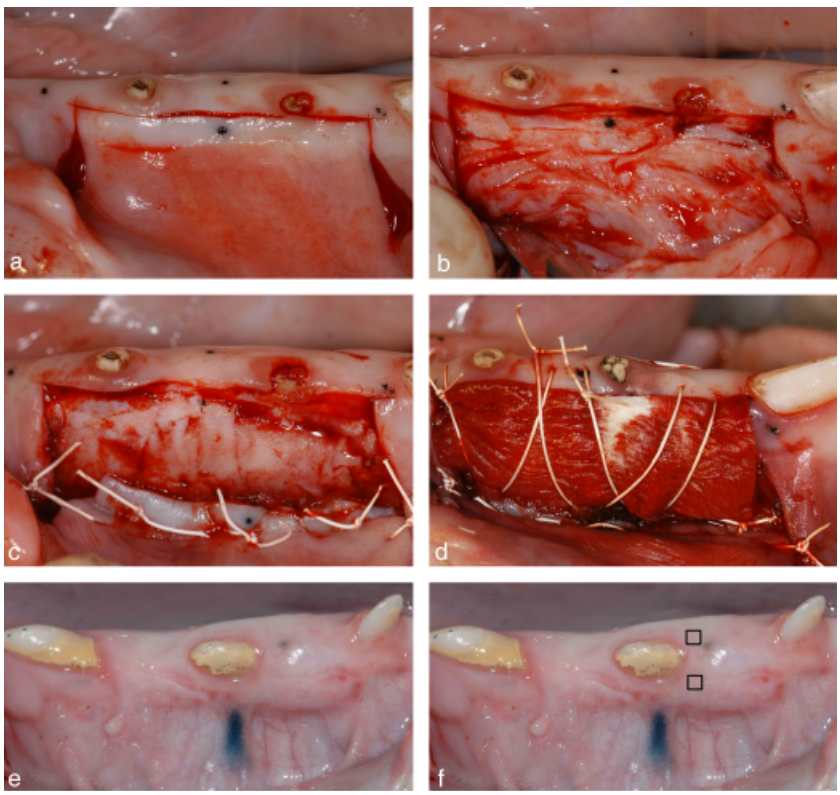

Fig. 2. (a) Defect preparation with a split-thickness flap. (b) Split-thickness flap has been elevated. (c) subepithelial connective tissue has been removed and the flap has been apically positioned and sutured. In the control sites, no further treatment was applied (d) collagen matrix has been placed on a denuded ridge defect and sutured in place. (e) Clinical situation (M1) at 6 months demonstrating a fully healed gingival tissue and revealing gain in keratinized tissue (f) same site as in (e) depicting squares used for measurements of colour differences between augmented tissue and host tissue.

test for independent data and using the Mac Nemar test for dependent data. For all tests, a *p*-value lower than 0.05 was considered as statistically significant.

Results

Clinical and macroscopic observations

The animals were healthy throughout the entire study period. No infection or any sign of local intolerance related to the surgery and the materials was observed.

A time effect was observed within each treatment group for the thickness and the width of the keratinized gingiva. The thickness of the gingiva increased in all groups between baseline and 1 month and decreased slightly between 1 and 6 months, resulting in an overall increase of 3.0 mm (SD ± 0.5 mm; control sites), 3.1 mm (± 1.0 mm; M2), and 3.5 mm (± 0.5 mm; M1) between baseline and 6 months (Fig. 3a, Table 1). The changes between the 3 time-points were statistically significant within each treatment group ($p < 0.05$). However, no statistically significant differences were observed among the three treatment modalities ($p > 0.05$).

The width of the keratinized tissue continuously increased between baseline and 6 months in all three groups, with mean values between 8.8 mm (± 2.0 mm; M1), 9.8 mm (± 2.0 mm; M2), and 10.7 mm (SD ± 3.3 mm; control sites) (Fig. 3b, Table 1). The changes were again statistically significant between the 3 time-points within each treatment group ($p < 0.05$). In contrast, no statistically significant differences were detected among the three treatment modalities ($p > 0.05$).

The reepithelization of the defect sites was almost completed at 1 month, ranging from 97 ± 5.5% (M2) to 99 ± 1.8% (both M1 and control), and completed in all sites in all animals at 6 months (Table 1).

The colour differences (ΔE) between augmented tissue and surrounding tissue revealed mean values of 4.4 for M2, 4.9 for M1, and 6.0 for controls. All these colour differences were above the clinically visible threshold of 3.7 (Johnston & Kao 1989, Jung et al. 2008). The differences in ΔE between M1 and controls amounted to 3.6, and between M2 and controls, ΔE was 2.2. These colour differences between treatments were not statistically significantly different ($p < 0.05$).

Histological observations (Fig. 4)

At 1 month (biopsies) (Fig. 4a).

All three treatment modalities showed active fibroconnective formation overlaid by a regular keratinized layer. Very slight and negligible inflammatory signs were noted with the two matrices. The presence of residual macrophages was related to the degradation process of the matrices. Remnants of matrix material were only found in sites treated with M2.

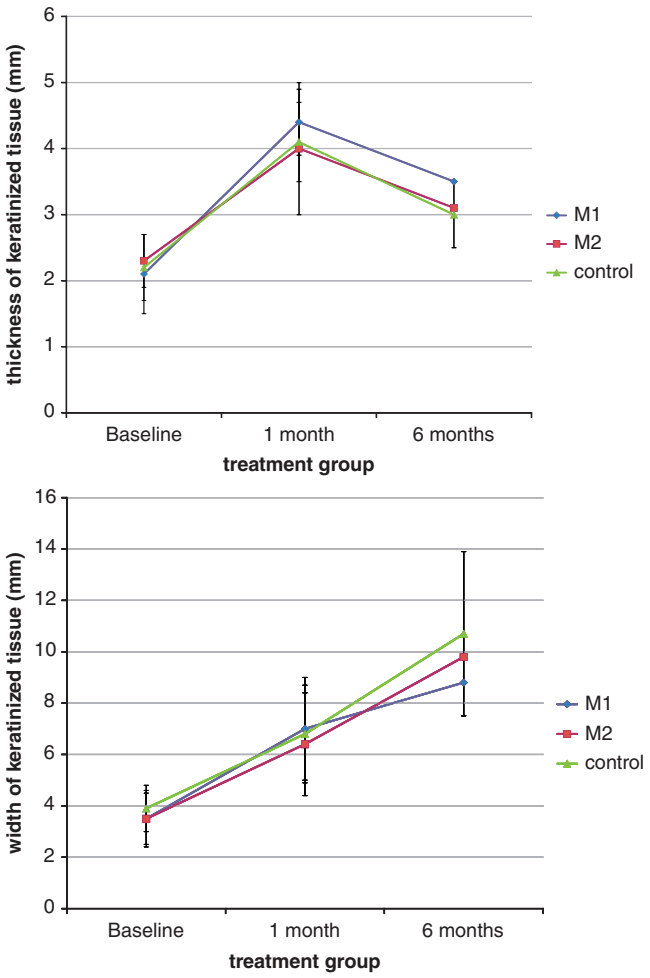

Fig. 3. Clinical measurements recorded and shown over time with respect to the thickness of keratinized tissue (a) and width of keratinized tissue (b) for all three treatment modalities (M1, M2, and control sites).

Table 1. Clinical measurements and observations for all three time-points and treatment modalities with means and standard deviations (SD)

Parameter	Group	Time-point		
		baseline	1 month	6 months
Thickness of keratinized gingiva (mean ± SD, in mm)	M1	2.1 ± 0.4	4.4 ± 0.6	3.5 ± 0.5
	M2	2.3 ± 0.7	4.0 ± 0.4	3.1 ± 1.0
	Control	2.2 ± 0.5	4.1 ± 0.6	3.0 ± 0.5
Width of keratinized gingiva (mean ± SD, in mm)	M1	3.5 ± 0.4	7.0 ± 1.1	8.8 ± 2.0
	M2	3.5 ± 1.1	6.4 ± 1.0	9.8 ± 2.0
	Control	3.9 ± 0.9	6.8 ± 1.9	10.7 ± 3.2
Reepithelialization (mean ± SD, in %)	M1	NA	99 ± 1.8	100
	M2		97 ± 5.5	
	Control		99 ± 1.8	

NA, not applicable.

At 6 months (specimens) (Fig. 4b and c).

All sites were completely healed, with signs of mature submucosal and epithelial tissues. Muscular fibres, new vessels, and signs of innervation were observed. The healing patterns (tissue regeneration) were not significantly different among the three groups. Neither inflammatory reactions nor residues of matrix material were found in sites treated with the two matrices at this later time-point.

Discussion

The clinical results demonstrated a safe integration of the newly developed collagen matrices into the surrounding tissue without any signs of inflammation. The thickness and width of the keratinized tissue increased significantly over 6 months in all groups, resulting in slightly more favourable results for M1 (compared with M2) with respect to the thickness of keratinized tissue and for M2 (compared with M1) with respect to the width of keratinized tissue. The histologic analysis revealed a healthy oral mucosa and only a very slight inflammatory reaction in collagen matrix sites.

The width of keratinized tissue increased over 6 months in all groups. The two collagen matrix groups were slightly less effective than the control sites. However, the control sites showed a greater variability, with a standard deviation that was 50% higher than M1 and M2 sites. It has been demonstrated earlier that an apically positioned flap alone can significantly increase the width of keratinized tissue (Fagan 1975, Marxer et al. 1982, Mohammadi et al. 2007). Moreover, the measurements in the present animal study also demonstrated an increase in the width between 1 and 6 months. This is in contrast to clinical studies showing limited changes between 1 and 6 months for sites treated with an apically positioned flap (Marxer et al. 1982) or with experimental collagen matrices (Sanz et al. 2009). The total gain in the width of keratinized tissue appears to be much greater compared with the outcomes of a recent systematic review, revealing a mean gain of 4.5 mm for an apically positioned flap with autogenous tissue (Thoma et al. 2009). Also, older clinical and experimental studies revealed contrasting results and a certain unpredictability with respect to the zone of keratinized gingiva that can be increased by periosteal retention or denudation of the alveolar bone (Bohannon 1962a, b, Karring et al. 1975a).

The thickness of keratinized tissue predominantly increased within the first month of healing, with mean values around 4.0 mm in all the groups. Subsequently, the thickness of keratinized tissue decreased slightly between 12% (M2), 17% (control sites), and 20% (M1) probably due to remodelling processes within the tissue. These findings correlate with a clinical study, demonstrating that an apically positioned flap can increase the thickness of the keratinized gingiva (Fagan 1975).

An excellent tissue integration of the two matrices without any signs of inflammation was observed. This is in contrast to an earlier study investigating ADMG (Wei et al. 2002). It has been shown that the use of ADMG to increase the width of keratinized tissue results in the presence of a large amount of elastic fibres, which are normally present in the alveolar mucosa and not in the keratinized tissue. No rete ridges were observed; instead, a flat interface with a frequent appearance of chronic inflammatory cells was detected. The degree of keratinization varied between ortho- and mainly para- or non-keratinized (Wei et al. 2002). The collagen matrices in the present study consisted of two functional layers: a cell occlusive layer on top and a porous structure underneath that should induce blood clot formation. This resulted in an orthokeratinized epithelium including the typical rete ridges in all matrix-treated sites. It is speculated that the collagen matrices served as a scaffold and allowed an overgrowth of an epithelium that was derived from the surrounding keratinized tissue. Underneath, the connective tissue part was similar to the one of a regular keratinized tissue. This observation is also supported by the clinical measurement of the grade of epithelization, which was mostly completed at 1 month, fully completed at 6 months, and without statistically significant differences between the three treatment modalities. Clinical data available for an apical positioning of the gingival margin suggest that the exposed periosteum contributes to the amount of keratinized tissue by acting as a graft itself (Fagan 1975). This clinical procedure can lead

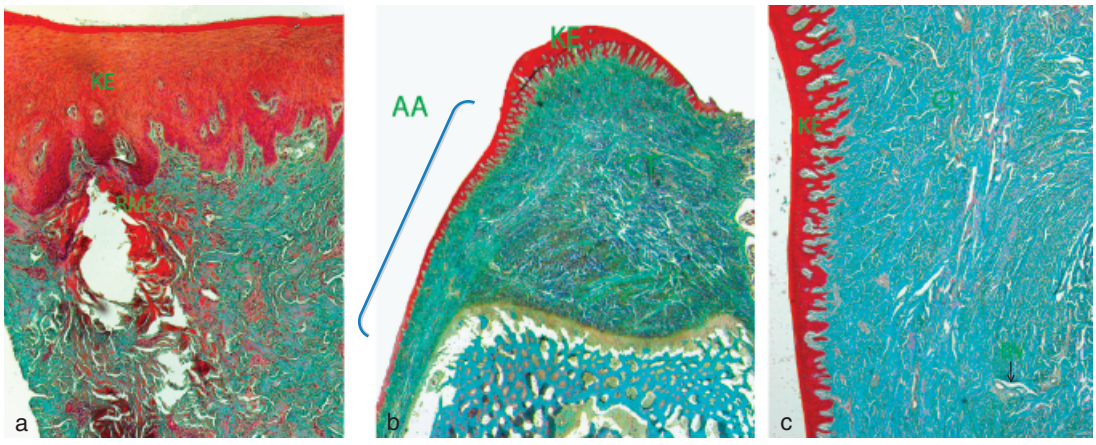

Fig. 4. Light micrographs of histological slides. (a) Biopsy at 1 month of M2 showing orthokeratinized epithelium (KE), connective tissue (CT), and remnants of M2 (RM2); original magnification of × 10. (b) and (c). Light micrograph at × 1.25 and × 5 original magnification demonstrating orthokeratinized epithelium (KE), mature connective tissue (CT), blood vessels (BV), and augmented area (AA). No remnant of M1 can be observed.

to an increase of keratinized tissue without placing a dressing on top of the periosteum (e.g. an autogenous graft) (Fagan 1975). The reepithelization following an apically positioned flap has been evaluated in a previous clinical study (Diedrich et al. 1972). This study revealed that 14 days after the surgery, the entire periosteum was covered with a granulation tissue. At 4 weeks postoperative, the healing was in general completed similar to that in the present study. At 16 weeks postoperatively, no clinically visible scar tissue was detectable in that clinical study. These clinical findings are comparable with the present animal study 6 months after surgery. Clinical results using either one of the treatment modalities revealed similar outcomes in the present study. However, resorption of the underlying alveolar bone and an increased patient morbidity have been reported when using an apically positioned flap alone (Pfeifer 1965, Ramfjord & Costich 1968). These issues are mainly due to a healing by secondary intention. It is expected that under clinical conditions in humans, the application of a topical dressing such as a collagen matrix could reduce the patient morbidity. As the present study was an animal experiment, these outcomes could not be evaluated.

Another important factor for an eventual clinical application of collagen-based matrices is the colour of the augmented tissue compared with the host tissue. Clinical studies demonstrated that the use of autogenous grafts increasing the width of keratinized tissue resulted in colour differences between augmented and surrounding tissue (Wei et al. 2000, McGuire & Nunn 2005, McGuire et al. 2008). This is mostly due to the fact that the transplanted tissue derived from the palate governs the extent of keratinization, whereas the epithelium is derived from the surrounding tissue (Karring et al. 1971, Karring et al. 1975b). In contrast, a variety of clinical studies investigating dermal substitutes revealed clinically more favourable results with respect to colour differences compared with autogenous grafts (Wei et al. 2000, McGuire & Nunn 2005, McGuire et al. 2008). In the present study, the colour differences between augmented areas and surrounding tissues were above the clinically visible threshold value of 3.7 for all the groups (Johnston & Kao 1989). However, the colour differences calculated between the different groups were below 3.7, indicating similar aesthetic outcomes for all groups. This favourable clinical observation is supported by a recent clinical study, reporting excellent colorimetric results when using similar collagen-based matrices (Sanz et al. 2009). The excellent aesthetics obtained using collagen matrices will likely be one of several clinical advantages over autogenous tissue. An additional benefit is the lack of a second surgical intervention that will not only save time but also reduce patient discomfort. An increased patient morbidity has been documented by clinical studies harvesting autogenous tissue from the palate (Farnoush 1978, Griffin et al. 2006). This observation is mostly due to the second surgical site (Griffin et al. 2006). Pain and general discomfort have been reported predominantly during the first 3 weeks post-surgery (Del Pizzo et al. 2002, Soileau & Brannon 2006). In addition, the quality and quantity of tissue that can be retrieved at the palate varies between patients (Soileau & Brannon 2006). Using a device from the shelf may help to solve the difficulties and problems associated with autogenous tissue in the future.

Conclusions

The clinical results demonstrated a safe integration of the experimental collagen matrices into the surrounding tissue without any signs of inflammation. The thickness and width of the keratinized tissue increased significantly over 6 months similar to an apically positioned flap. Within the limits of this animal study, all three treatment modalities may be used to increase the width of keratinized tissue.

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Clinical Relevance

Scientific rationale for the study: Prototype collagen matrices were examined as a replacement for autogenous tissue to increase the width of keratinized tissue. The present study was designed in order to evaluate efficiency, safety, local tolerance, and tissue integration.

Principal findings: The clinical results demonstrated a safe integration of the experimental collagen matrices into the surrounding tissue without any signs of inflammation. The thickness and width of the keratinized tissue increased significantly over 6 months.

Practical implications: Within the limits of this animal experiment, an apically positioned flap with or without collagen matrices may be used to increase the width of keratinized tissue.