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Comparison of ATMP gingiva and skin substitutes and their *in vitro* wound healing potentials

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Abstract

Skin and oral mucosa substitutes are a therapeutic option for closing hard-to-heal skin and oral wounds. In this study we describe bi-layered skin and gingiva substitutes cultured under identical conditions, which are compliant with current European regulations for advanced therapy medicinal products. We present in vitro mode of action methods to i) determine viability: epithelial expansion, proliferation (Ki-67), metabolic activity (MTT-assay); ii) characterize skin and gingiva substitutes: histology and immunohistochemistry, and iii) determine potency: soluble wound healing mediator release (ELISA). Both skin and gingiva substitutes consist of metabolically active autologous reconstructed differentiated epithelium expanding from the original biopsy sheet on a fibroblast populated connective tissue matrix (donor dermis). Gingival epithelium expanded 1.7 fold more than skin epithelium during the 3 week culture period. Also, the percentage of proliferating Ki-67 positive cells located in the basal layer of the gingiva substitute was > 1.5 fold higher than in the skin substitute. Keratins 16 and 17, which are upregulated during normal wound healing, were expressed in both the skin and gingiva substitutes. Notably, the gingiva substitute secreted higher amounts of key cytokines involved in mitogenesis, motogenesis and chemotaxis (IL-6 > 23-fold, CXCL8 > 2.5-fold) as well as higher amounts of the anti-fibrotic growth factor HGF (> 7-fold) compared to the skin substitute. In conclusion, whilst addressing viability, characterization and potency of the tissue substitutes, important intrinsic differences between skin and gingiva were discovered which may explain in part the superior quality of wound healing observed in the oral mucosa compared to skin.
incorporated with regards to viability, characterization and potency (mode of action) under cGMP.

In this study we identify the mode of action in vitro of the second generation SS and GS and in doing so we describe important intrinsic differences between SS and GS. These tissue specific differences may explain in part the superior quality of wound healing generally observed in oral mucosa compared to skin [16–19].

Material and methods
Skin and gingiva tissue
Healthy human skin and gingiva biopsies were obtained after informed consent from patients undergoing corrective abdominal plastic surgery (skin) and dental implant surgery (gingiva). Skin and gingiva tissue was used anonymously and in accordance with the “Code for Proper Use of Human Tissue” as formulated by the Dutch Federation of Medical Scientific Societies (www.fmivv.nl), and following procedures approved by the VU University medical centre institutional review board. No clinical signs of inflammation or scar were present in the tissues used (determined by surgeon or dentist). The gingiva biopsies (epithelium and lamina propria) were obtained from the edentulous area. After tooth extraction, the extraction site was left to heal for at least 3-6 months before an implant was placed. Prior to placing the implant a 6 mm diameter biopsy was removed. The biopsy was sent to the research laboratory within 24 hours after harvesting and was further biopsied in the research laboratory into 3 mm diameter biopsies. Abdominal skin tissue was received with epidermis, dermis and subcutaneous fat present. The fat was removed and then 3 mm diameter biopsies were taken. Therefore, both skin and gingiva biopsies used for experiments were 3 mm diameter and approximately 2-3 mm thick.

Construction of skin and gingiva substitutes
Skin and gingiva substitutes (SS and GS respectively) were constructed essentially as described previously [13,14]. For this study, tissue from 7 gingiva donors and 7 skin donors was received in the culture facility. With the exception of one skin and two gingiva donors (infection in incoming biopsies), tissue from the donors was successfully cultured as determined by > 1 mm visible epithelial outgrowth from the original epithelial sheet; a stratified epithelium being present; and > 50% confluent fibroblasts in the transwell at the point in time which the epithelium and fibroblasts are combined. The average age of gingiva donors was 69 years (SD ± 1.6) and of skin donors it was 36 years (SD ± 7.4). Gingiva donors were mostly male (4/5), while skin donors were mostly female (5/6). Results described in this manuscript are derived from 5 GS donors and 6 SS donors.

In brief, a single batch of SS or GS was constructed from two 3 mm diameter skin or gingiva biopsies and one piece of acellular human donor dermis (1.5 x 2.5 cm²). Acellular dermis was prepared from glycerol-preserved donor skin (Euro Tissue Bank, Beverwijk, the Netherlands). Glycerol and dead donor cells were removed by repeated washing over a period of approximately 10 days in Dulbecco’s PBS (1x) ATMP-ready (PAA, Pasching, Austria) with 50 μg/ml gentamicin (Centrafarm, Etten-Leur, The Netherlands) at 37°C. Remnants of dead epidermis were gently scraped off using a spatula until only the white acellular dermis remained. The acellular dermis, with basement membrane intact (collagen IV, collagen VII, BP180 and HSPG expression), was stored at 4°C in Dulbecco’s PBS (1x) ATMP-ready until used to construct SS and SG [13]. Next, two epithelial sheets were separated from the connective tissue of two biopsies from a single skin or gingiva donor after overnight incubation on dispase II (Roche, Mannheim, Germany) at 4°C. Intact epithelial sheets were placed with the differentiated side upwards onto the basement membrane side of the acellular donor dermis and cultured at the air-liquid interface on keratinocyte medium consisting of Dulbecco’s modified Eagle medium (DMEM) (SAFC, Zwijndrecht, The Netherlands) / Ham’s F-12 (SAFC) (3:1) containing 5% Fetal-Clone III (Thermo Scientific, Waltham, MA USA), 2.5 μg/ml isoprenalin (Monico SPA, Venice, Italy), 0.5 μg/ml Solu-Cortef (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 μg/ml Actrapid (NovoNordisk, Bagsvaerd, Denmark), 50 μg/ml gentamicin and 2 ng/ml LONG EGF (Repligen, Lund, Sweden).

Primary fibroblasts were isolated by incubating the connective tissue from one of the biopsies in dispase (BD biosciences, Breda, The Netherlands) / collagenase (Nordmark, Uetersen, Germany) for 2 hours at 37°C. The entire digest from the biopsy containing the skin or gingiva fibroblasts was transferred to a transwell (0.4 μm pore size, cat nr 3450, Costar, Corning Incorporated, New York, USA) and the adherent fibroblast cells were cultured for 7-10 days in DMEM containing 5% Fetal Clone III and 50 mg/ml gentamycin. After 7-10 days of culturing the primary fibroblasts and epithelial sheet apart, the acellular donor dermis containing the epithelial sheet was placed onto the fibroblasts in order to allow fibroblast migration into the reticular side of the dermis. The SS and GS were further cultured air exposed in keratinocyte medium (see above), but with only 1% Fetal-Clone III and without gentamicin.

Culture medium was renewed twice a week and SS and GS were harvested three weeks after initiating culture from the original biopsy. At this point the substitutes would otherwise be applied to an ulcer or oral lesion. Culture supernatant collected over the last 3-4 days of culturing was stored at -20°C until further analysis by ELISA. Harvested cultures were formalin-fixed and paraffin embedded using standard methods. The culture medium and method of culture was accepted by the Dutch authorities as being fully compliant with European ATMP requirements.

Histology, immunohistochemistry and MTT assay
Paraffin embedded tissue sections (5 μm) were used to assess histology (haematoxylin and eosin staining (HE)) and for immunohistochemical staining of keratins, Ki-67, vimentin, loricrin, involucrin and SKALP (skin derived anti-leukocyte protease). Epithelial outgrowth from the biopsy was analyzed on HE stained sections with a Zeiss Axioscope 20 microscope and NIS-Elements AR 3.2 software.
Immunohistochemical staining was performed essentially as described previously [14]. For staining of K6, K10, K13, K16, K17, Ki-67 and vimentin, antigen retrieval was performed by immersion of the slides in 0.01 M citrate buffer (pH 6.0) for 15 min at 100°C, followed by slow cooling to room temperature during 3 hours. For loricrin, involucrin and SKALP endogenous peroxidase was inhibited by 20 minute incubation in 0.3% H2O2 in methanol solution. Subsequently, loricrin was pre-incubated with goat serum (Dako, X0907) (Glostrup, Denmark). All sections were incubated for 1 hour with primary antibodies; K6: clone KA12, Cat nr 61090, (Progen Biotechnik, Heidelberg, Germany); K10: clone DE-K10, Cat nr 11414, Progen Biotechnik); K13: clone 1C7, Cat nr MON3017, Monosan (Uden, The Netherlands); K16: clone LL025, Cat nr MONX10691, Monosan; K17: clone e3, Cat nr MONX10692, Monosan; Ki-67: clone Mib1, Cat nr M7240, Dako; loricrin: clone AF-62, Cat nr PRB-145P-100, Covance Inc. (Princeton, NJ, USA); involucrin: clone SY5, Cat nr NCI-INV, Novocastra (Leica Biosystems, Newcastle, United Kingdom); SKALP: clone TRAB2O, Cat nr HM2062, Hycult Biotech (Uden, The Netherlands); Vimentin: clone V9, Cat nr M0725, Dako. For K13 staining incubation with post-antibody blocking and incubation with powervision-HRP solution was performed. Slides for all stainings were incubated with Envision (Dako, K4001), except for loricrin, which was incubated with goat-anti rabbit-biotin (Dako, E0432) and streptavidin-HRP (Dako, P0397) both 30 minutes.

The proliferation index is expressed as the number of Ki-67 positively stained nuclei/total number of basal epidermal cells x 100%. From three different regions of each section approximately 100 basal cells were counted and then averaged. Metabolic activity was analyzed using the MTT assay with 3 mm biopsies taken from the visible outgrowth of the epithelium. Biopsies were incubated in a 2 mg/ml MTT solution (Sigma-Aldrich) for 2 hours at 37°C, and transferred to an isopropanol/HCL solution (3:1). The next day the color intensity was measured at 570 nm.

Quantification of cytokine and growth factor secretion

For the quantification of IL-6, CCL2, CCL5, CCL27, CCL28, VEGF, HGF and bFGF in culture supernatants, ELISA reagents were used in accordance with the manufacturer’s specifications. Commercially available paired ELISA antibodies and recombinant proteins obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA) were used. For CXCL8, a Pelipair reagent set (Sanquin, Amsterdam, The Netherlands) was used. Since SS and GS are derived from two 3 mm punch biopsies growing on a single piece of acellular dermis (1.5 x 2.5 cm) with 12.5 ml culture medium, results are expressed as pg/ml derived from the 12.5 ml culture supernatant and 3.75 cm2 (1.5 x 2.5 cm) tissue substitute. This method of analysis was accepted by the Dutch authorities as a means of expressing standardized potency of an autologous tissue substitute designed for clinical applications. The detection limits of the ELISAs were 30 pg/ml.

Statistical analysis

All data are presented as mean ± standard error mean. Results represent SS and GS data obtained from 6 skin donors (N=6) and 5 gingiva donors (N=5) respectively. Therefore each N represents a separate donor and also an independent experiment. SS were cultured in duplicate from each donor and the two values were averaged to a single value per donor, but due to the limited amount of tissue available, GS were cultured in single fold from each donor. Differences between skin and gingiva substitutes were calculated with two-tailed, unpaired T-tests. Statistics were calculated in GraphPad Prism (San Diego, CA, USA). Differences were considered significant when *P < 0.05, **P < 0.01, ***P <0.005.

Results

Since the SS and GS were produced under identical culture procedures they provide an excellent tool to investigate similarities and differences between the mode of action of the two tissue substitutes. We present in vitro mode of action data concerning i) viability: epithelial expansion, proliferation (Ki-67), metabolic activity (MTT assay); ii) characterization: histology and immunohistochemistry; and iii) potency: soluble wound healing mediator release (ELISA).

Viability: Gingiva substitutes show enhanced epithelial outgrowth and proliferation compared to skin substitutes

Metabolic activity, which correlates to cell viability, was determined with an MTT assay. No difference was observed between the metabolic activities of 3 mm diameter punch biopsies removed from SS and GS, and all values obtained were above those of dead acellular donor dermis clearly indicating that the tissue substitutes were viable (Figure 1D).

Both SS and GS consisted of a viable differentiated epithelium growing out from the original 3 mm diameter epithelial sheet and a fibroblast populated human connective tissue matrix. Similar to the native biopsies, the SS had a multilayered orthokeratized epithelium, while the GS had a parakeratinized epithelium in which the terminally differentiated cells in the upper layers retained remnants of nuclei. Rete ridge formation in the SS and GS was not as pronounced as in the native biopsies (Figure 1A, B and C). This is in line with the previously reported 1st generation SS and GS [14].
Since epithelialization is achieved by keratinocyte migration and proliferation, first the histology and extent of outgrowth from the skin and gingival epithelial sheets over the connective tissue matrix after the three week culture period was compared. The histology of the mid-section adjacent to the original epithelial sheet was analyzed separately from the outermost migrating epithelial front and both were compared to the intact healthy native biopsy (Figure 1C). Histology of the mid-section of the SS closely resembled that of the native biopsy, with a stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The mid-section of the GS also resembled the native biopsy with the cells within the upper most differentiated layers retaining remnants of nuclei. Whereas SS epithelium had approximately the same number of living cell layers as the native tissue biopsy, GS had clearly less cell layers in the midsection compared to the native tissue biopsy. In contrast to the mid-sections, the migrating fronts of both SS and GS contained no well-defined differentiated cell layers. Also, the GS migrating front was thinner and more extended than the SS migrating front, which is indicative of a faster epithelial outgrowth (Figure 1C). Indeed the gingival epithelium expanded 1.7 fold more than skin epithelium during the three week culture period (Figure 1D). Furthermore, the percentage of proliferating Ki-67 positive cells was > 1.5 fold higher in GS compared to SS (Figure 1D and Figure 2). Taken together, our findings on epithelial migration and proliferation correlate to the higher turnover and wound closure (epithelialization) capacity of gingiva compared to skin.

Characterization: Keratins 16 and 17 are strongly expressed in expanding skin and gingiva epithelium

Differential keratin expression is a characteristic of epithelial tissues derived from different body locations. For example keratin 16 is expressed in native gingiva, but is absent in native skin (Figure 2). Furthermore, keratins 16 and 17 are described to be upregulated during wound healing since they disrupt the rigid structure of the epithelium thus allowing keratinocytes to migrate over each other and over the connective tissue matrix [20]. Notably, both keratins were expressed in the midsections of the SS as well as GS. Whereas K17 was also expressed in the migrating fronts of both SS and GS, K16 was clearly absent in the most undifferentiated frontal cells in both the SS and GS (Figure 2). Keratins 6, 10, 13, loricrin and involucrin and SKALP showed differential expression in the SS and GS which was representative of the original biopsy tissue and in line with our previous findings with 1st generation SS and GS [13,14] (Table 1).
Potency: Gingival substitutes secrete higher amounts of wound healing mediators than skin substitutes

The potency of both SS and GS can be ascribed to the type and amount of secreted mediators that stimulate wound healing and vascularization. Therefore cytokines (Figure 3A) and growth factors (Figure 3B) secreted by SS and GS that have been described to play a role in wound healing (Table 2) were compared.

| Table 1: Summary of (immuno)histological comparison between skin and gingiva substitute |
|-----------------------------------------------|----------------|----------------|
| **Characteristics**                          | **Skin substitute** | **Gingiva Substitute** |
| Keratin 6                                   | SS, SG           | SB-U           |
| Keratin 10                                  | SS, SG           | SB-1*          |
| Keratin 13                                  | Absent           | +/- SB**       |
| Keratin 16                                  | SB               | SB             |
| Keratin 17                                  | SB               | SB             |
| Loricrin                                    | SG               | SB-U           |
| Involucrin                                   | SG               | SB-U           |
| SKALP                                        | SG               | SB-U           |
| Ki67                                         | BL               | BL             |

BL, basal layer; SG, stratum granulosum; SS, stratum spinosum; SB, suprabasal; SB-U, upper suprabasal layers; SB-I, intermittent expression in suprabasal layers; SKALP, skin derived anti-leukocyte protease. * present in two GS only; ** present in three GS only

Figure 2: Histology and immunohistochemistry of native skin and gingiva biopsies and skin and gingiva substitutes. Representative Ki-67 and keratin 16 and 17 staining is shown for native biopsy tissue, the midsection and the migrating front (end section) of SS and GS. Scale bar = 100 µm

| Table 2: Cytokines and growth factors involved in wound healing |
|---------------------------------------------------------------|----------------|
| **Name**                                                     | **Function in wound healing** | **Reference** |
| Interleukin 6 (IL-6)                                         | Immune response during infection and after trauma | [34,35] |
| CXCL8                                                       | Mediator in innate immune response | [23,34] |
| CCL2 / MCP-1                                                | Macrophages, T-cell and mast cell chemoattractant | [35] |
| CCL5 / RANTES                                                | Fibroblast migration | [36] |
| CCL27 / CTACK                                                | Effector cell recruitment to sites of epithelial injury | [37] |
| Hepatocyte growth factor (HGF)                              | Keratinocyte migration and proliferation | [30,35] |
| Vascular endothelial growth factor (VEGF)                    | Stimulation of vasculogenesis and angiogenesis | [34] |
| Basic fibroblast growth factor (bFGF / FGF-2 / FGFβ)        | Fibroblast proliferation, keratinocyte migration and proliferation | [34] |

GS secreted higher amounts than SS of key cytokines involved in mitogenesis, motogenesis and chemotaxis (IL-6 > 23-fold, CXCL8 > 2.5-fold) as well as higher amounts of the anti-fibrotic growth factor HGF (> 7-fold) (Figure 3). No differences were found between GS and SS with regards to chemokines CCL2, CCL5, CCL27 and growth factor VEGF secretion. CCL28 and bFGF were undetectable in both SS and GS culture supernatants.
higher keratinocyte migration and proliferation, as well as enhanced secretion of wound healing and anti-fibrotic mediators (IL-6, CXCL8 and HGF) in GS when compared to SS.

The autologous SS and GS have been designed for the clinical setting in which biopsies are taken from the patient to be treated. Each SS and GS is 1.5 x 2.5 cm² and therefore can be used to cover 3.75 cm² wound surface. For larger wounds, more biopsies are required in ratio to the wound size. We have previously shown that large chronic ulcers (> 150 cm²) can successfully be treated with the 1st generation SS. Oral wounds are generally smaller than skin wounds and therefore will require less biopsies to construct sufficient GS pieces to close the wound [13–15].

It was not possible to determine directly the efficacy of fibroblast migration into the reticular side of the dermis since the individual SS and GS were too small to isolate and quantify cells migrating into the dermis. However quality controls during culture confirmed fibroblast viability (> 50 confluent after approximately 1 week culture) at the time point when fibroblasts are combined with the epithelial sheet growing on the dermis. During the following 2 weeks (3 weeks culture total), fibroblasts migrated into the dermis. Many fibroblasts remain in the lower regions of the dermis, with few cells migrating into the upper regions. However, since we have previously shown that soluble mediators HGF, IL-6, CCL2, CCL5 and CXCL8 are secreted by SS only when fibroblasts are present [12], and since we show that vimentin is detectable in both SS and GS, we can conclude that viable fibroblasts as well as epithelial cells are present in the SS and GS.

Clinical studies show that gingiva has a higher number of epithelial cell layers and a higher turnover than skin and also has a faster rate of wound closure. Our results clearly show that the increased proliferation and migration capacity of gingiva compared to skin is an intrinsic property of the epithelium, and that our GS and SS closely mimic corresponding native healing tissues. However, since we did not observe an increased number of epithelial cell layers in GS compared to SS, we can conclude that the transition of proliferating keratinocytes into terminally differentiating keratinocytes and the following stratification into a multilayered epithelium is regulated in part by extrinsic environmental factors, possibly for example by soluble mediators present in saliva [21].

Figure 3: Wound healing mediators secreted by skin and gingiva substitute

A: Cytokine secretion (IL-6, CXCL8, CCL2, CCL5 and CCL27) and B: growth factor secretion (VEGF, HGF) by SS and GS is shown. Protein levels in the culture supernatants were determined by means of ELISA. Background amounts of cytokines and growth factors within the fibroblast culture medium were below the detection limits of the ELISAs (data not shown). Bars represent means ± SEM of N=6 skin donors for SS and N=5 gingiva donors for GS. * P <0.05, ** P <0.01

Discussion

In general, oral wounds heal more rapidly and with better final scar quality than skin wounds [16–19]. In this study we characterized and compared the mode of action of SS and GS which were cultured under identical procedures and which are fully compliant with the current European regulations for ATMPs. Taken together, our results show that gingiva tissue is intrinsically more primed towards enhanced wound healing compared to skin, given the higher keratinocyte migration and proliferation, as well as enhanced secretion of wound healing and anti-fibrotic mediators (IL-6, CXCL8 and HGF) in GS when compared to SS.

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Upon injury, keratin 16 and 17 are strongly induced in post-mitotic cells at the wound edge. It is thought that these keratins may promote reorganization of the cytoplasmic array of keratin filaments [20]. This is an event that precedes the onset of keratinocyte migration into the wound site. In the mid-section of SS, which mimics actively proliferating and migrating epidermis, K16 and K17 were highly expressed in the suprabasal cell layers, similar to the corresponding region in GS. However, we found that in the very outermost migrating front of both SS and GS where only one or two undifferentiated keratinocyte cell layers are present, K16 was absent. In line with these findings, an elevated level of K16 in mice has been shown to partially impair keratinocyte migration, although the mechanism is unknown [22]. Taken together, these findings suggest that K16 and K17 enable keratinocytes to migrate over each other, but K16, in contrast to K17, may not be involved in keratinocyte migration...
over the connective tissue matrix. Minor discrepancies (regarding GS proliferation and K17 expression) were observed when comparing our results with our previous study [14]. These can most probably be attributed to the fact that in our previous study gingiva was obtained from molar tooth extractions whereas in this study gingival biopsies were obtained from the edentulous area prior to placing dental implants. The SS and GS were further extensively characterized with the aid of immunohistochemistry. Close correlations were found between the tissue substitutes and their native tissues with regards to epithelial keratin expression, loricrin and involucrin and SKALP expression which again emphasized the specific intrinsic differences between skin and gingiva.

In order to determine the potency of the tissue substitutes, the secretion of cytokines, chemokines and growth factors described to play a role in wound healing was determined (Table 2). Secretion of wound healing mediators IL-6 and CXCL8 by GS was much higher than by SS, again supporting the greater intrinsic healing capacity of gingiva compared to skin. For CXCL8 and IL-6 literature is conflicting. There is evidence that elevated levels of CXCL8 stimulate keratinocyte proliferation in vitro [23,24], but the opposite, inhibition of keratinocyte proliferation has been described as well [25]. Furthermore elevated levels of CXCL8 have been described to contribute to delayed wound healing since CXCL8 was increased in non-healing human thermal wounds compared to healing wounds. High amounts of CXCL8 and IL-6 were detected in wound extracts of non-healing ulcers [26]. However, it has also been described that TNFα stimulates oral keratinocytes to produce more CXCL8 and IL-6 than skin keratinocytes [27,28]. In general, IL-6 and CXCL8 are pleiotropic in nature, exhibiting increases and decreases according to the homeostatic environment.

Interestingly, gingiva not only heals faster than skin, but also heals with negligible final scarring [17,19,29], although it is currently unknown why. HGF has been shown to have an anti-fibrotic effect and it also acts as mitogen and motogen [30]. HGF has been described to act anti-apoptotic on endothelial cells, whilst at the same time it promotes myofibroblast apoptosis. These properties would clearly be expected to increase the quality of a scar and suggests that the increased amount of HGF secreted by GS compared to SS may contribute to the intrinsic property of gingiva to heal with superior scar quality. Indeed in the pilot study in which three tooth extraction sites were treated with the 1st generation GS, the oral lesions healed with negligible scarring [14].

Surprisingly, we show that GS are able to secrete the skin specific chemokine CCL27, whereas the mucosa homologue CCL28 was undetectable. Both chemokines are lymphocyte chemo attractants [31] and CCL28 has high homology with CCL27 [32]. Recently we have shown in another gingiva substitute model (reconstructed epithelium on a fibroblast populated collagen hydrogel) that gingiva can indeed secrete CCL27 and that it is also inducible with TNFα, albeit to a much lower extent than in skin equivalents [33]. The reason why CCL28 was undetectable is currently unknown, however it is possible that it is directly internalized by other cells in the vicinity such as fibroblasts.

In this study we describe for the first time a SS and GS cultured under identical conditions, which are fully compliant with the current European regulations for ATMPs. In addition to strict culture methods and quality controls, ATMP regulations now require information on the mode of action of the final product. In this study we have presented in vitro methods which were accepted by the Dutch authorities. Taken together, our information on mode of action has high-lighted intrinsic differences between the two tissues which could be related to superior oral mucosa healing.
Reference List


