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Supporting Information

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Microphysiological Modeling of Gingival Tissues and Host-Material Interactions Using Gingiva-on-Chip

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TITLE

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SUPPLEMENTARY METHODS

1. Cell Culture

Donor-derived primary gingival fibroblasts were isolated from the gingival tissues attached to healthy, non-carious impacted third molars after institutional review board approval (DSRB No. 2018/00256) as previously described [1, 2]. Primary human gingival fibroblasts (passage 5-7) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Immortalized human OKF6/TERT2 oral keratinocytes [3] (a kind gift from Dr. J. Rheinwald, Harvard Medical School, Boston, MA) were maintained as described previously [4, 5] under basal conditions using keratinocyte serum-free medium (K-SFM, ThermoFisher Scientific) supplemented with 25 µg/mL bovine pituitary extract (ThermoFisher Scientific), 0.2 ng/mL epidermal growth factor (EGF, ThermoFisher Scientific) and 0.09 mM calcium chloride (Sigma).

2. Biofabrication of gingival equivalents under static and flow conditions

Gingival equivalents on-chip (flow) and insert (static) were fabricated through a three-stage process comprising of the fabrication of gingival connective tissue equivalents (CTEs), seeding of keratinocytes followed by culture at air-liquid interface (***Figure 2A***). To fabricate gingival CTEs (or lamina propria-like equivalents) on-chip and insert, gingival fibroblasts were encapsulated within 80 µL and 180 µL of human fibrin-based mucosal matrix respectively as previously described [5, 6]. Briefly, to fabricate gingival CTEs of 80 µL volume, gingival fibroblasts (2 x 10^4 cells) were suspended in 10 µL of human fibrinogen (10 µg/mL, Merck Millipore) conjugated with 2.5 µL of polyethylene glycol, succinimidyl glutarate terminated (PEG, 10kDa, 0.6 mg/mL, Sigma Aldrich). The cell-PEG-fibrinogen solution was polymerized with equal volume of crosslinking solution containing thrombin (6.25 UN/mL) in 20 mM calcium chloride (Sigma Aldrich). For on-chip cultures, the mucosal matrix was seeded onto the culture chamber within the microfluidic device (***Figure 2A***). Similarly, for insert cultures, the mucosal matrix was seeded into 12-well ThinCert™ cell culture inserts (pore size 1 µm, polyethylene terephthalate membrane, Greiner Bio-one). The gingival CTEs were cultured for 4 days using a low-serum media containing 1x SITE supplement (Sigma-Aldrich), ascorbic acid (10 mg/mL, Sigma-Aldrich), hydrocortisone (50 µg/mL, Sigma-Aldrich), basic fibroblast growth factor (10 ng/mL, Miltenyi Biotec), aprotinin (20 KIU/mL, MP Biomedicals), 1% penicillin-streptomycin.

In the second phase (after four days), oral keratinocytes (3 x 10^5 cells/cm^2) were seeded on top of the mucosal matrix and cultured for 2 days under submerged conditions using a low-serum media containing 1x SITE supplement, ascorbic acid (10 mg/mL), hydrocortisone (50 µg/mL), basic fibroblast growth factor (10 ng/mL), EGF (5 ng/mL), aprotinin (20 KIU/mL), and 1% penicillin-streptomycin (***Figure 2A***). In the third phase, to initiate epithelial stratification and differentiation, the keratinocyte-seeded mucosal matrix was cultured at an air-liquid interface for 14 days using a low-serum media containing 1x SITE supplement, ascorbic acid (10 mg/mL), hydrocortisone (50 µg/mL), aprotinin (20 KIU/mL), and 1% penicillin-streptomycin (***Figure 2A***).

3. Histology and immunostaining

Tissue equivalents were fixed with 10% neutral buffered formalin (Sigma) and histoprocessed for embedding into paraffin blocks. Deparaffinized tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) or immunostained for oral epithelial (cytokeratins CK5, CK10, CK13, CK19), basement membrane (collagen-IV. laminin-V), and connective tissue (vimentin, collagen-I, fibronectin) markers as detailed in ***Supplementary Table 2***. Briefly, the tissue sections were subjected to epitope recovery (121ºC in 0.01M citrate buffer (pH6.0) for 20 minutes) followed by blockage of non-specific staining and incubation with relevant primary antibodies (listed in ***Supplementary Table 2***) overnight at 4ºC. After washing, the sections were incubated with appropriate secondary antibodies for 45 minutes, washed and nuclei labelled using DAPI.

4. Image analysis

Microphotographs of tissue sections (5 µm thick) stained with H&E and immunostained for Ki67 expression were captured using a brightfield microscope (Nikon Eclipse E600 equipped with NIS-Elements software). Microphotographs of tissue sections immunostained with fluorescent antibodies were captured using laser scanning confocal microscopy (Olympus FluoView™ FV1000), and images processed using Fiji/Image J (NIH, USA) and Imaris software (Oxford Instruments).

Viable epithelial thickness was quantified as previously described [7] using a line tool (NIS-Elements software, Nikon) that measured the length from the basement membrane to the upper surface of stratum granulosum. The measurements were captured at 4-6 random points for each imaging filed and 3-4 random fields along the length of the epithelium for each tissue sample. All data are presented as the mean ± standard deviation (SD) of atleast three independent biological tissues.

Ki67 proliferation index was computed as previously described [7] by counting the number of Ki67-positive nuclei across the length of the epithelium at 4-5 random fields for each tissue section. Since the basement membrane in the full-thickness gingival equivalents is flat, the Ki67 proliferation index was normalized as the number of Ki67-positive nuclei per mm length of the epithelium. All data are expressed as the mean ± SD of atleast three independent biological tissues.

Intensity and area of expression of stratified epithelial cytokeratin (CK14), early and late keratinocyte markers CK10 and loricrin respectively were quantified using Fiji/Image J (NIH, USA). Intensity of expression was measured as the mean grey values within a selected region of interest (ROI) of a standardized area across all samples and expressed in arbitrary units (a.u.). To quantify the area of expression, the area of the epithelium positive for the marker was outlined and measured using the polygon tool (Fiji/Image J). For loricin (marker of late differentiation marker and epithelial barrier) and collagen IV (basement membrane marker), the number of breaks in their expression was counted and normalized to unit length of the epithelium along the basement membrane (unit length of 400 µm refers to the average length of the epithelium per imaging field). All data are expressed as the mean ± SD of atleast three independent biological tissues.

5. TUNEL staining for apoptosis

Induction of apoptosis following exposure to various test substances was assessed using the DeadEnd fluorometric Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay kit (Promega, USA) based on the manufacturer's instructions. Briefly, the deparaffinized tissue sections were treated with 4% formaldehyde for 15 mins and washed with PBS. To permeabilize the tissue sections, we incubated the slides in proteinase K at room temperature for 10 mins, followed by PBS wash. Slides were equilibrated with buffer for 5-10 mins, labelled with terminal deoxynucleotidyl transferase reaction mix, and incubated in the dark humified chamber for 60 mins. The reaction was stopped and counterstained

with DAPI and mounted. Images were captured using a confocal fluorescence microscope (Olympus Fluoview FV-1000).

6. Cell viability assay (MTT assay) on tissue equivalents

Cellular viability of the tissue equivalents following exposure to test compounds was assessed based on modified OECD test guidelines (TG439) using CellTiter96® Non-Radioactive Cell Proliferation Assay (Promega, USA). Briefly, the medium was removed and replaced with 1 mg/ml MTT dye solution on the basolateral side and incubated for 3 h at 37°C. MTT dye solution was removed, and the tissues were washed thrice with sterile PBS. For extraction of the formazan crystals, the tissues were wiped with clean absorbent paper, immersed into 2 mL of solubilization solution, and incubated for 2 h with gentle shaking at 125 rpm. Optical density of the blue formazan extraction solution was measured using a 96-well plate spectrophotometer (MultiskanGo, ThermoFisher) at 570 nm. Blank corrected OD values of tissue treated with negative control (PBS) was assumed as 100% viability. The viabilities of the positive control (1% SLS) and mouthwashes were normalized with respect to the negative control. All data were presented as mean ± SD of three independent biological tissues. The mucosal irritation potential of the test substance was classified based on the prediction model presented in the OECD TG439. Test substance was classified as irritant, if the relative viability is below 50% with respect to the negative control.

7. Lactate dehydrogenase (LDH) cytotoxicity assay

The culture media following the incubation period after exposure to the test substances were collected, and total LDH released by tissue equivalents were quantified using Promega CytoTox-One homogenous membrane integrity assay kit (Promega). Briefly, the culture media (50 µL) were transferred to a black walled 96-well plate and 50 µL of CytoTox-One reagent was added in all the wells. The samples were mixed and incubated at room temperature for 10 minutes followed by fluorescence measurement at excitation/emission 560nm/590nm. The fluorescence values were blank corrected and normalized with respect to the negative control. All data were presented as mean ± SD of three independent biological tissues.

8. Computation of permeation kinetics

The amount of lidocaine HCl and articaine HCl in the perfusates collected from the receptor compartments was quantified using spectroscopic measurement of peak absorbance at 221 nm and 272 nm, respectively [8, 9]. Calibration plots were constructed for lidocaine HCl and articaine HCl, which were linear (R^2=0.999) in the range of 0.195-20 µg/ml.

Permeation parameters were estimated from cumulative amount profiles using the infinite dose transport model described previously [9]. Briefly, the steady-state flux (J_{ss}; µg/cm^2.h) was determined from the slope of the best-fit linear portion of the cumulative amount profiles of the dental anaesthetics permeated per unit diffusion area versus time, which is expressed as

$$Q_{ss}(t) = K_p . C_d \, (t - t_{lag}) \qquad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$J_{ss} = \frac{C_d . K . D}{L} = C_d . K_p \qquad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where, Q_{ss} designates the steady-state cumulative amount, K_p -permeability coefficient (10^{-3} cm/h), C_d - applied donor concentrations of the tested drug, t - time, t_{lag} - lag time, K - partition coefficient, D - diffusion coefficient and L - thickness of the membrane/tissue model. All values are expressed as the mean ± standard error of three or more biological tissues.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Permeation parameters of the dental anaesthetics

	Gingiva-on-Chip	Ulcer-on-Chip	Sig
Lidocaine	*n = 3*	*n = 3*	
Surface dose (donor) *[mg.cm^{-2}]*	1.25	1.25	
Steady-state Flux J_{ss} *[µg.cm^{-2}.h^{-1}]*	10.4 ± 3.3	42.6 ± 11.2	*0.03*
Permeability coefficient: k_p *[mm.h^{-1}]*	0.1 ± 0.0	0.4 ± 0.1	*0.03*
Cumulative amount Permeated *[µg.cm^{-2}]*	44.2 ± 12.5	170.7 ± 40.0	*0.02*
Articaine	*n = 4*	*n = 4*	
Surface dose (donor) *[mg.cm^{-2}]*	2.45	2.45	
Steady-state Flux J_{ss} *[µg.cm^{-2}.h^{-1}]*	17.6 ± 4.7	73.7 ± 17.7	*0.01*
Permeability coefficient: k_p *[mm.h^{-1}]*	0.2 ± 0.0	0.7 ± 0.2	*0.01*
Cumulative amount Permeated *[µg.cm^{-2}]*	74.3 ± 16.8	256.8 ± 50.6	*0.003*

Supplementary Table 2. Details of primary antibodies used in the study

Marker	Clone & Species	Epitope recovery	Dilution factor	Source & Catalogue no.
CK5	Rabbit Polyclonal (EPR1600Y)	Citrate buffer [a]	1:500	Abcam (ab75869)
CK10	Mouse Monoclonal (DE-KRT10)	Citrate buffer [a]	1:100	Thermo Scientific (MA5-13705)
CK14	Mouse Monoclonal (LL001)	Citrate buffer [a]	1:1	In-house [b]
CK13	Mouse Monoclonal (KS-IA3)	Citrate buffer [a]	1:100	Novus Biologicals (NBP1-22777)
CK19	Mouse Monoclonal (LK2P)	Citrate buffer [a]	1:1	In-house [b]
Collagen-I	Rabbit Polyclonal	Citrate buffer [a]	1:600	Abcam (ab34710)
Collagen-IV	Mouse Monoclonal (CIV22)	Citrate buffer [a]+ Proteinase-K [c]	1:500	Dako (M0785)
Fibronectin	Rabbit Polyclonal	Citrate buffer [a]	1:1200	Sigma Aldrich (F3648)
Ki67	Mouse Monoclonal (MIB-1)	Citrate buffer [a]	1:200	Dako (M7240)
Laminin-V	Mouse Monoclonal (D4B5)	Citrate buffer [a]+ Proteinase-K [c]	1:100	EMD Millipore (MAB19562)
Vimentin	Mouse Monoclonal (V9)	Citrate buffer [a]	1:500	Novocastra

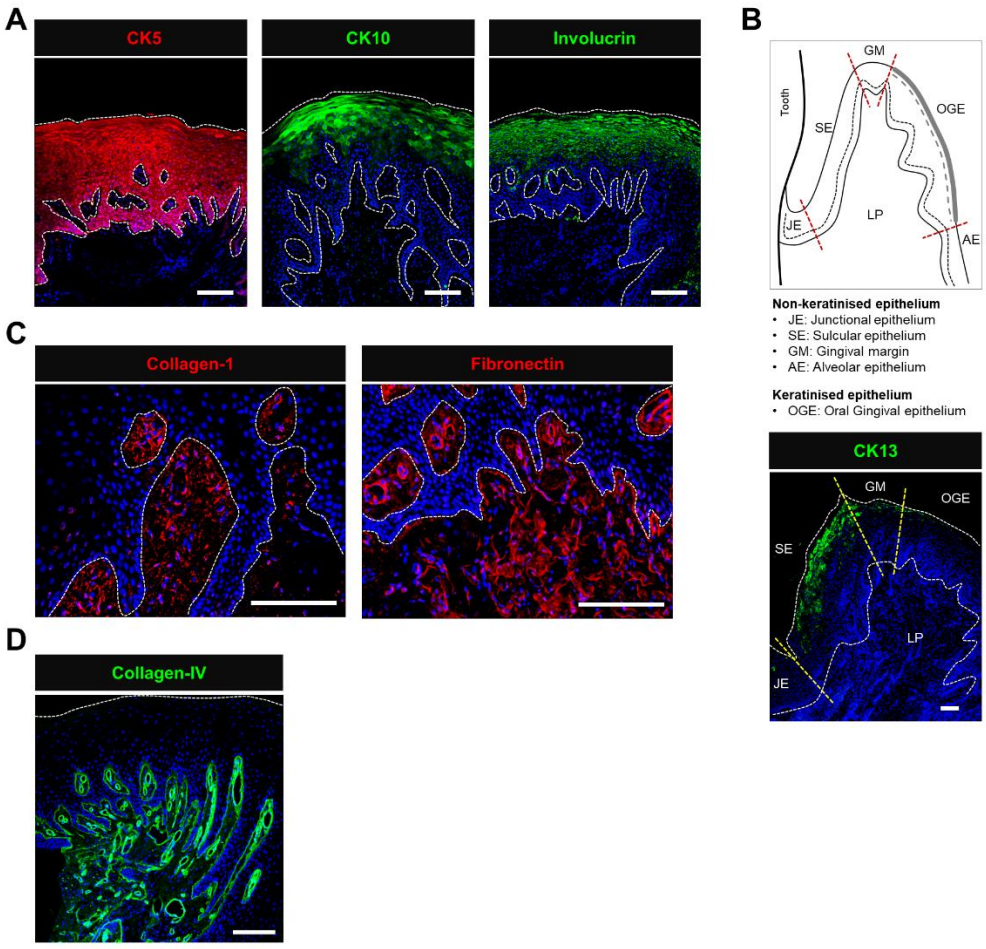
Note:

[a] The slides were heated at 121ºC in 0.01M citrate buffer (pH6.0) for 20 minutes in a pressure-based antigen retriever and cooled to room temperature for at least 2 hours before blocking and antibody incubation.

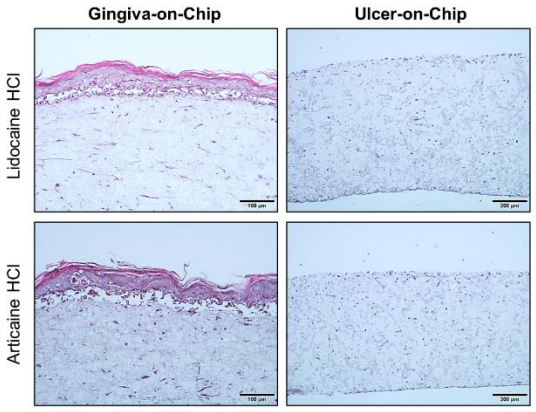
[b] Kind gift from Declan Lunny and Birgitte Lane, Epithelial Biology Laboratory, Institute of Medical Biology, A*STAR, Singapore.

[c] After epitope recovery with citrate buffer, the slides were incubated with Proteinase-K (20 µg/ml) for 15 minutes at room temperature.

SUPPLEMENTARY DATA



Supplementary Figure S1. Immunostained sections of human gingiva showing the expression of (**A,B**) stratified epithelial markers (CK5, CK10, involucrin and CK13), (**C**) matrix proteins (collagen-I, fibronectin) and (**D**) basement membrane marker collagen-IV (Scale bar: 100 µm).



Supplementary Figure S2. Integrity of the gingiva-on-chip and ulcer-on-chip tissues following culture and dental anaesthetic permeation on the microfluidic device.