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Title

Gingival 3D organotypic cell culture

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07/09/2016

Time frame

Visit to PMI and assessment of digital images: June 13-15th 2016.
First draft: 10th July 2016
Second draft: 17th July 2016
Third draft: 30th July 2016

Contents

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1. Objectives

To assess the morphological aberration in the gingival 3D organotypic cultures (EpiGingival™) following conventional cigarettes (reference cigarette – 3R4F, provided by Kentucky University) smoke or test item aerosol exposure.

2. Evaluation method

The 3D organotypic culture samples (EpiGingival, MatTek, Ashland, MA, USA) tested comprised a 6.5 mm disc, bisected, and processed as FFPE and stained with Haematoxylin and Eosin. The scanned disc assessed comprised two 6.5 mm lengths of the organotypic culture (diameter of the disc), which adding the two together resulted in a 13 mm length of material available for assessment.

No established parameters were available. A guidance chart provided for buccal organotypic culture (provided by PMI) was referred to because of the similarity between buccal and gingival culture models with the main difference being that masticatory mucosa (e.g. gingiva) is keratinised whereas oral lining mucosa (e.g. buccal) is not keratinised.

A gingival histological analysis table (Table 1) was therefore constructed based partially on findings observed in an oral (buccal) mucosal analysis table available. This was modified after assessment of control culture samples and exposed sampled. The thickness of each layer within the epithelium and their distinctiveness (e.g. sharp delineation versus blurred) was also recorded.

Observations and comments were entered into an Excel chart (Supplementary file 1_ Blinded data) at the time of assessment. All samples were blinded at that time except for the incubator control samples, identified as such in order to be used as a base line (considered as 'normal' morphology) against which to compare encountered changes. All samples were rearranged once unblinded (supplementary file 2_Unblinding codes gingival).

3. Sample included in the analysis

Outline provided by PMI (Filippo Zanetti), slightly modified.

The study is designed to assess the effects of a 3-day repeated exposure of the gingival tissue cultures to conventional cigarettes or a test item. The exposure was performed from a Monday (day 1) to a Wednesday (day 3). In each day the cultures were exposed to smoke from 10 cigarettes or aerosol from 10 test items during 28 minutes.

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the day of collection (all groups except group 12, 13 14, 15). PBS was removed and replaced with fresh one before each exposure. The intention of adding PBS on the apical side was to mimic the presence of liquid (saliva) in the mouth, which moistens the gingival tissue *in vivo*. Incubator controls were also covered with PBS.

There were three phases (main phases 1-3) of the study, each with a control, samples exposed to conventional cigarette smoke and to aerosol from the test item, diluted to different nicotine concentrations). There was also a Dose range finding section (DRF), where a broader range of test item aerosol concentrations was tested.

Main phase 1: PBS was used to cover the apical side of all groups except groups 12-15.

Main phase 2: PBS was used to cover the apical side of all groups except groups 12-15.

Main phase 3: PBS was used to cover the apical side in all groups.

DRF phase: PBS was used to cover the apical side in all groups

For a given phase, the exposed samples were collected all on a Thursday (Day 4, 24 h after the last exposure) and processed for histological analysis (H&E staining).

The thirty (30) control samples were assessed after days 2 (Group 1), 3 (Group 2), 4 (Group 3) and 5 (Group 4).

Samples were provided as follows (Unblinding provided prior to the analysis).

Main phases (P-1: Phase 1, P-2: Phase 2, P-3: Phase 3)

Phase 1 = 42 samples **(9 controls, 9 conventional cigarettes with PBS, 12 test item with PBS, 12 no PBS)**
Phase 2 = 42 samples **(9 controls, 9 conventional cigarettes with PBS, 12 test item with PBS, 12 no PBS)**
Phase 3 = 24 samples **(3 controls, 9 conventional cigarettes with PBS, 12 test item with PBS)**

No PBS: Main phases, N = 24

Exposure type	Phase	Dose / phase	Harvest day 2	Harvest day 3	Harvest day 4	Harvest day 5
Incubator controls	P-1	N/A	A1, A2, A3			
	P-1			B1, B2, B3		
N = 21	P-1				C2, C3, C4	
	P-2			A1, A2, A3		
	P-2				B1, B2, B3	

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Conventional cigarettes. Main phase with PBS (Nicotine mg/L) With PBS N = 27	P-1	0 (Sham)			D1, E1, F1	
	P-2				C1, D1, E1	
	P-3				C1, D1, E1	
	P-1	49.4			D2, E2, F2	
	P-2				C2, D2, E2	
	P-3				C2, D2, E2	
	P-1	84.6			D3, E3, F3	
	P-2				C3, D3, E3	
	P-3				C3, D3, E3	
Test item Nicotine mg/L) With PBS N = 36	P-1	0 (Sham)			K1, L1, M1	
	P-2				C4, D4, E4	
	P-3				C4, D4, E4	
	P-1	14.4			K2, L2, M2	
	P-2				C5, D5, E5	
	P-3				C5, D5, E5	
	P-1	54.6			K3, L3, M3	
	P-2				C6, D6, E6	
	P-3				C6, D6, E6	
	P-1	100.4			K4, L4, M4	
	P-1				C7, D7, E7	
	P-1				C7, D7, E7	
Conventional cigarettes. Main phase with PBS (Nicotine mg/L) Without PBS N = 12	P-1	0 (Sham)			G1, H1, J1	
	P-2				F1, G1, H1	
	P-1	49.4			G2, H2, J2,	
	P-2				F2, G2, H2	
Test Item: main phases (Nicotine mg/L) Without PBS N = 12	P-1	0 (Sham)			N1, P1, Q1	
	P-2				F3, G3, H3	
	P-1	14.4			N2, P2, Q2	
	P-2				F4, G4, H4	

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Dose range assessment / finding (DRF) = 45 samples.

Exposure type	Coded ID	Other description
Incubator controls (N = 9)	A1, A2, A3	Harvested Day 2
	B1, B2, B3	Harvested Day 3
	C1, C2, C3	Harvested Day 4
Conventional cigarettes. (Nicotine mg/L) (N=18)		
0 (Sham)	D1, E1, F1	-
3.11	D2, E2, F2	-
8.25	D3, E3, F3	-
15.4	D4, E4, F4	-
89.1	D5, E5, F5	-
201	D6, E6, F6	-
Test item Nicotine mg/L) (N = 18)		-
0 (Sham)	G1, H1, J1	-
2.18	G2, H2, J2	-
5.84	G3, H3, J3	-
11.5	G4, H4, J4	-
79.2	G5, H5, J5	-
147.2	G6, H6, J6	-

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4. Assessment results

4.1. Comment on 3D organotypic culture being assessed

Comments made are based upon the histological appearance of incubator control samples, detailed below (4.4) The 3D organotypic cell culture (EpiGingival) sections appeared histologically virtually identical to normal human Gingival mucosa[1]. It comprised keratinised, stratified squamous epithelium, as described in native human mucosa. The culture sat on an artificial membrane rather than a basement membrane and had the following four layers from the membrane towards the surface (Figure 1):

A) Stratum basale (basal layer)
B) Stratum spinosum (prickle layer)
C) Stratum granulosum (granular layer) and
D) Stratum corneum (keratinised layer).

Keratinisation represents the differentiation of keratinocytes in the stratum granulosum into non-viable (non-vital) superficial squamous cells where their nuclei become pyknotic and eventually disappear. Parakeratosis is when nuclei persist, often due to cell damage and increased number of cells from the stratum granulosum becoming keratinised.

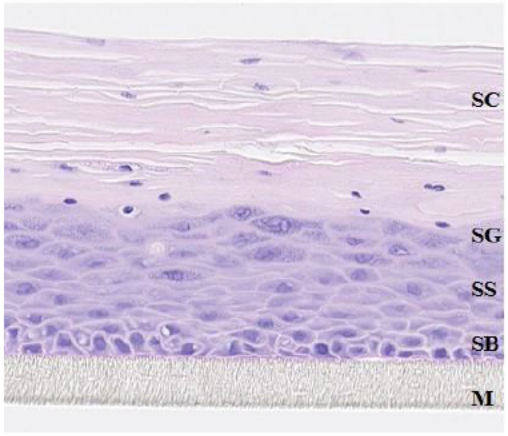

Figure 1. Histology of the gingival organotypic model used in the study.
M, membrane, SB, Stratum basalis, SS, Stratum spinosum, SG, Stratum Granulosum, SC, Stratum corneum.

4.2. General observations on 3D organotypic culture samples

Incubator samples were regarded as 'normal controls' for purposes of the study. Their appearance and features were regarded as a baseline for the analysis.

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phases and DRF).

Uniform features, which were observed across these samples were:

A) Thickness was generally uniform across each of the individual samples.
B) Apoptosis / single cell degeneration was seen as single cells with retracted cytoplasm and a pale or dark compact nucleus
C) Sharp boundary between each layer, particularly the stratum granulosum and stratum corneum. There were pyknotic nuclei several cells thick within the lower stratum corneum. Keratohyaline granules, which characterise the stratum granulosum, were generally confined to that layer.
D) Splitting of the stratum corneum was a constant finding and this could show variable separation making assessment of thickness determination difficult. Areas where there was less separation were chosen, and in some instances, gaps were not included in the thickness measurement (see (A) above).
E) Stratum corneum could be more compact (usually with more retained nuclei – parakeratin which was always present particularly in the lower levels of the stratum corneum) or loose with few nuclei (true orthokeratin) usually more superficially.
F) The tissue usually retained its adherence to the artificial membrane, except where there could be some artefactual detachment e.g. due to folding.
G) Some sampled displayed corrugation, where the tissue seemed at regular intervals to lift from the slide. This was often seen on test samples also. This became an issue in some cases because the scanner focussed on one plane and if this was the plane with less material, the majority was out of the plane of focus (this was an issue in only several test cases only, please see below).
H) Apoptosis was noted.
I) Only occasional mitoses were noted.

Refer to section 4.4 below for more detail.

4.3. Assessment of all blind non-control (Exposure) samples:

The findings in all blinded samples (excluding the controls which were known) could be distilled into essentially five patterns. Including the control tissue appearance there were six patterns as in the table:

Finding	Explanation	Representative picture of pattern

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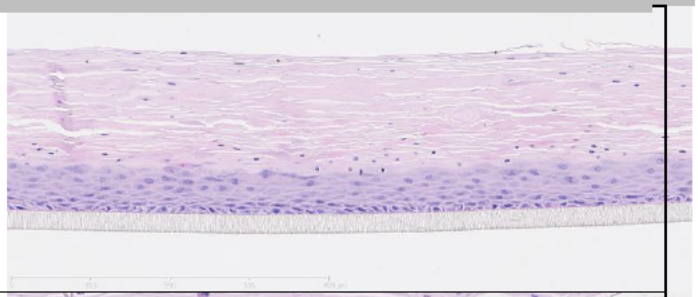
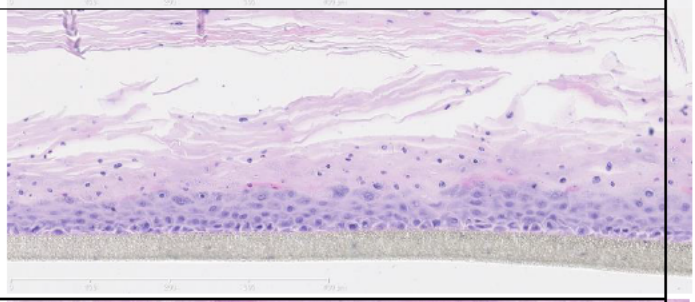
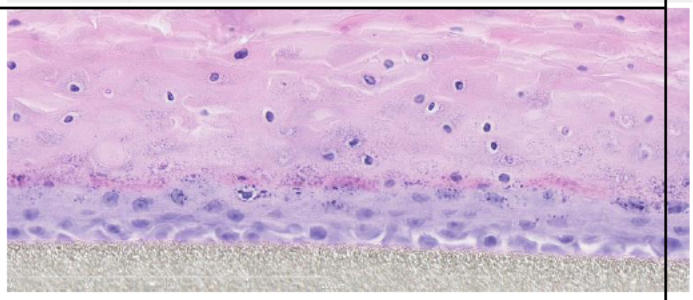
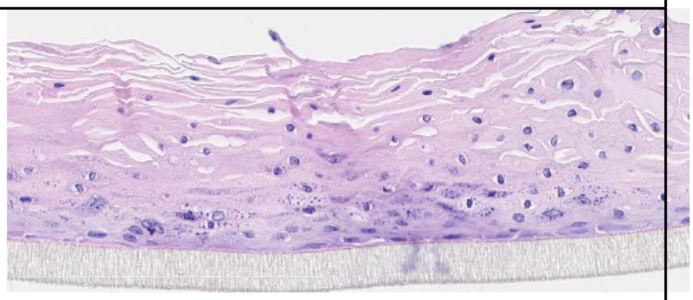
Pattern 0 Normal (control)	Well delineated layers, occasional mitoses and apoptosis noted.	
Pattern 1	Mild alteration leading to blurring of the distinction between the stratum granulosum and the stratum corneum. This resulted in increased keratinisation of the stratum granulosum and pyknosis of nuclei so the sharp cut off was diminished.	
Pattern 2	More pronounced blurring of and loss of distinction between stratum granulosum and the stratum corneum with or without hypergranulosis and with keratohyaline granules staddling both the stratum granulosum and the stratum corneum. The Stratum spinosum (prickle layer) may be slightly thinned (atrophy), but shows no overt apoptosis.	
Pattern 3	Pronounced blurring of and loss of distinction between stratum granulosum and the stratum corneum with typically hypergranulosis and with keratohyaline granules staddling both the stratum granulosum and the stratum corneum. The Stratum spinosum (prickle layer) typically shows atrophy, but shows no overt apoptosis.	

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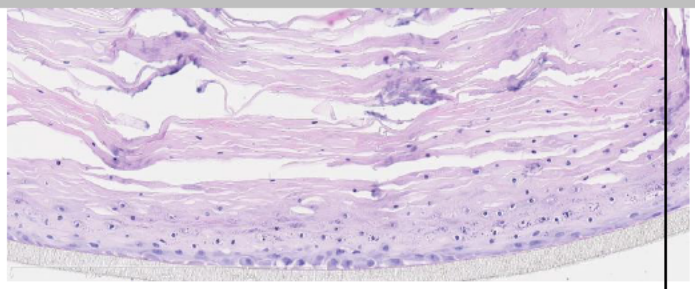
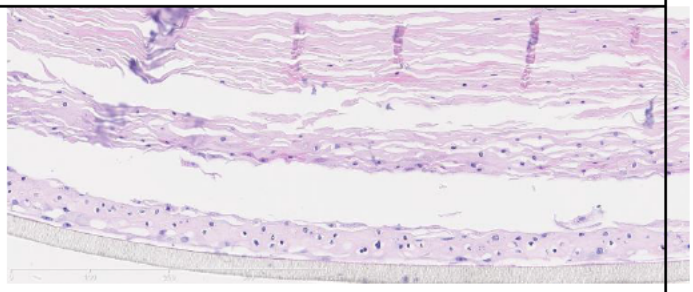
Pattern 4	Almost complete loss of the stratum spinosum (atrophy) with keratinisation beginning soon after the stratum basale (basal layer) with only 2-4 basal cell thickness before keratinisation. Apoptosis/karyorhexis/pyknosis is increased up to 15-20 cells per mm length but is difficult to quantitate. There is typically coarse hypergranulosis.	
Pattern 5	Complete loss of the stratum spinosum and basal layer (atrophy) with keratinisation / maturation extending down to the membrane. Apoptosis/karyorhexis/pyknosis is increased up to 20+ cells per mm length. There may or may not be hypergranulosis and keratohyaline granules may be absent.	

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4.4. Findings observed for each group:

NB: 1 mm = 1000 µm

Incubator Control samples (all phases and DRF) N=30

Control samples were merged for each day of harvest.

Finding /Day	2 (N =6)	3 (N = 11)	4 (N = 10)	5 (N = 3)
Tissue thickness average (µm)	250	270	270	316 µm
Stratum granulosum thickness average (µm)	53	37	32	25 µm
% stratum corneum of total thickness	58 % (average)	70 % (average)	78 % (average)	81 % (average)
Apoptosis	up to 11 cells per length of 13 mm (average)	up to 7 cells per length of 13 mm (average)	up to 2.5 cells per length of 13 mm (average)	up to 2.7 cells per length of 13 mm (average)
Mitosis	one across three cases	our in total across eleven cases	four in total across ten cases	six in total across three cases (range 2-4)
Tissue appearance pattern type	0	0	0	0

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Conventional cigarettes: Main phases with PBS, N = 27

Finding /nicotine concentration (mg/ L)	**0 (Sham) (N = 9)**	**49.4 (N = 9)**	**84.6 (N = 9)**
Samples analyzed (codes)	P-1: D1, E1, F1; P-2: C1, D1, E1; P-3: C1, D1, E1	P-1: D2, E2, F2; P-2: C2, D2, E2; P-3: C2, D2, E2	P-1: D3, E3, F3; P-2: C3, D3, E3; P-3: C3, D3, E3
Tissue thickness average (µm)	284	287	283
Stratum granulosum thickness average (µm)	32	76	38
% stratum corneum of total thickness	79% (average)	95 % (average)	100% (average)
Apoptosis	up to 2.3 cells per total length of 13 mm (average)	up to 20 cells per 1 mm (average)	up to 30 cells per 1 mm (average)
Mitosis	@2 in total across three cases	Scattered above basal layer, not quantified.	2-3 recognised in several cases only
Atypia	minor reactive in 2 cases	Reactive / degenerative atypia in all	Reactive / degenerative atypia in all
Pattern type	0 or 1	4	5
Comment	Several cases have keratin pearls, several slight blurring of granular layer boundaries (pattern 1)	Reduced maturation, parakeratosis, some loss of cell order. Perinuclear vacuoles. Three cases, all from phase 1, show some epithelial disintegration and splits within epithelium and from membrane). Basal layer reactive and thinned	Reduced maturation, parakeratosis. Scattered suprabasal mitoses. Some loss of cell order. Essentially, the basal layer disappeared, and whole epithelium appeared keratinized.

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Test item: Main phases with PBS, N = 36

Finding /nicotine concentration (mg/ L)	**0 (Sham) (N = 9)**	**14.4 (N = 9)**	**54.6 (N = 9)**	**100.4 (N = 9)**
Samples analyzed (codes)	P-1: K1, L1, M1; P-2: C4, D4, E4; P-3: C4, D4, E4	P-1: K2, L2, M2; P-2: C5, D5, E5; P-3: C5, D5, E5	P-1: K3, L3, M3; P-2: C6, D6, E6; P-3: C6, D6, E6	P-1: K4, L4, M4; P-2: C7, D7, E7; P-3: C7, D7, E7
Tissue thickness average (µm)	273	285	293	283
Stratum granulosum thickness average (µm)	50	62	54	56
% stratum corneum of total thickness	82 % (average)	81% (average)	85% (average)	86% (average)
Apoptosis	1.6 cells per total length of 13 mm (average)	0.8 cells per total length of 13 mm (average)	0.8 cells per total length of 13 mm (average)	difficult to quantify, increased parakeratosis/pyknosis
Mitosis	2-3 in several cases only	few-'frequent' in several cases	Not noted	Not noted
Atypia	No	No	Some in pattern 3	Mild in most
Pattern type	0-1	1-2	Varied: 1, 2 & 3	Phase 2 & 3: pattern 3, one 4
Comment	Mild reactive basal changes. Few keratin pearl(s).	Mild hypergranulosis. Few keratin pearl(s).	Mild hypergranulosis. Few keratin pearl(s).	Phase 1 cases were pattern 1-2, one almost normal. Some atrophy noted.

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No PBS: Main phases, N = 24

Finding /nicotine concentration (mg/ L)	**0 (Sham) (N = 6)**	**49.4 (N = 6)**	**0 (Sham) (N = 6)**	**Test item 14.4 (N = 6)**
Samples analyzed (codes)	P-1: G1, H1, J1; P-2: F1, G1, H1	P-1: G2, H2, J2, P-2: F2, G2, H2	P-1: N1, P1, Q1; P-2: F3, G3, H3	P-1: N2, P2, Q2; P-2: F4, G4, H4
Tissue thickness average (µm)	262	248	245	248
Stratum granulosum thickness average (µm)	33	53	29	28
% stratum corneum of total thickness	66% (average)	94% (average)	67% (average)	71% (average)
Apoptosis	3 cells per total length of 13 mm (average)	15-20 cells per 1 mm (average)	1.2 cells per total length of 13 mm (average)	1.2 cells per total length of 13 mm (average)
Mitosis	several basal mitoses in two cases	Suprabasal mitoses noted on one case.	Not noted	Not noted
Atypia	No	Yes	No	No
Pattern type	0	4-5	0	0
Comment	Slightly thinner stratum corneum noted (reduced keratin layer).	Atrophy, parakeratosis.	Thinner stratum corneum noted (reduced keratin layer).	Relatively thinner stratum corneum noted c.f. comparable (reduced keratin layer).

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Dose range finding (DRF) = 45 samples.

Controls: DRF, N = 9 (included in general assessment of controls above)

Conventional cigarettes**: DRF, N = 18**

Finding /nicotine concentration (mg/ L)	**0 (Sham) (N=3)**	**3.11 (N=3)**	**8.25 (N=3)**	**15.4 (N=3)**	**89.1 (N=3)**	**201 (N=3)**
Samples analyzed (codes)	D1, E1, F1	D2, E2, F2	D3, E3, F3	D4, E4, F4	D5, E5, F5	D6, E6, F6
Tissue thickness average (µm)	318	372	423	357	277	278
Stratum granulosum thickness average (µm)	36	47	60	85	67	0 (loss of granules)
% stratum corneum of total thickness	79% (average)	84% (average)	83% (average)	80% (average)	96% (average)	86% (average)
Apoptosis	2 cells per total length of 13 mm (average)	1.3 cells per total length of 13 mm (average)	0.3 cells per total length of 13 mm (average)	1.3 cells per total length of 13 mm (average)	30 cells per 1 mm (average)	60-80 cells per total length of 13 mm (average)
Mitosis	Not noted	Not noted	Not noted	one basal mitosis in one	Not noted	Not noted
Atypia	No	No	No	No	Yes in all	Yes in all
Pattern type	0-1	0-1	1-2	1-2	4, and mostly 5	4-5
Comment	Nil	One out of focus (scanned on thin corrugations)	One half of one case out of focus. Keratin pearl noted.	Nil	Nil	Possible focal dysplasia in two cases

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Test item: DRF, N = 18

Finding /nicotine concentration (mg/ L)	**0 (Sham)**	**2.18**	**5.84**	**11.5**	**79.2**	**147.2**
Samples analyzed (codes)	G1, H1, J1	G2, H2, J2	G3, H3, J3	G4, H4, J4	G5, H5, J5	G6, H6, J6
Tissue thickness average (µm)	330	273	418	310	333	307
Stratum granulosum thickness average (µm)	42	42	92	48	63	50
% stratum corneum of total thickness	81% (average)	81% (average)	81% (average)	80% (average)	84% (average)	83% (average)
Apoptosis	1.7 cells per total length of 13 mm (average)	1.7 cells per total length of 13 mm (average)	2 cells per total length of 13 mm (average)	1.7 cells per total length of 13 mm (average)	2 cells per total length of 13 mm (average)	No cells noted per total length of 13 mm (average)
Mitosis	Not noted	Not noted	Not noted	Not noted	Basal mitosis in one case	Not noted
Atypia	Reactive	Reactive	Reactive	Reactive	Reactive	Reactive
Pattern type	0-1	0-1	1-2	1-2	1-2	1-2
Comment	One case partially out of focus, but histological evaluation was still possible.	Nil	Nil	Nil	Keratin pearl in one	Nil

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4.5. Summary of findings

Incubator Control samples: N = 30

The thickness of the culture seemed to increase over time, mostly observed for the stratum corneum. In contrast, the thickness of the stratum granulosum (granular layer) diminished, perhaps reflecting the increased keratinisation. Apoptosis was greater at day 2 and diminished over time. Mitoses were sparse and did not seem to particularly differ over time. It is possible apoptosis occurs initially and the cellular changes disappear over time. The reason for this is unclear.

Exposure samples:

Conventional cigarettes: Main phases with PBS, N = 27

The sham samples most often resembled the incubator controls (pattern 0), but several displayed changes designated as pattern 1, with slight blurring of the boundary between the granular (SG) and neighbouring layers (SS and SC). The samples exposed to a nicotine concentration of 49.4 mg/l showed almost complete loss of the stratum spinosum (atrophy) with keratinisation beginning soon after the stratum basale (basal layer) with only 2-4 basal cell thickness before keratinisation. Apoptosis/karyorhexis/pyknosis was increased but difficult to quantitate. There was typically coarse hypergranulosis (pattern 4). The samples exposed to a nicotine concentration of 84.6 mg/l showed complete loss of the stratum spinosum and basal layer (atrophy) with keratinisation / maturation extending down to the membrane. Apoptosis/karyorhexis/pyknosis was increased. Keratohyaline granules varies from increased to even absent (pattern 5).

Test item: Main phases with PBS, N = 36

The sham samples most often resembled the incubator controls (pattern 0), but with several displayed changes designated as pattern 1, with slight blurring of the boundary between the granular (SG) and neighbouring layers (SS and SC). There were progressive changes across increasing nicotine concentrations, with increased blurring of the layers, and hypergranulosis. The samples exposed to a nicotine concentration of 0 mg/l (Sham) showed either an appearance similar to the incubator controls, or only slight alteration (patterns 0-1). The samples exposed to a nicotine concentration of 14.4 mg/l showed increasing loss of distinction between the layers of the epithelium (patterns 1-2). The samples exposed to a nicotine concentration of 54.6 mg/l showed a range of changes from minor to blurred layers and mild atrophy (patterns 1, 2 and focal changes of pattern 3), whereas samples exposed to a nicotine concentration of 100.4 mg/l showed blurring of

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the layers, from mild to more prominent atrophy and degenerative nuclear changes (pattern 3 and in one pattern 4).

No PBS: Main phases, N = 24

Conventional cigarettes: main phases (N = 12)

The sham samples displayed pattern 0, but thinner stratum corneum than shams with PBS. The exposed samples were similar to the same nicotine concentration with PBS, with slightly more hypergranulosis than seen with PBS for same degree of change.

Test Item: main phases (N = 12)

The sham samples displayed pattern 0, but thinner stratum corneum than shams with PBS. The exposed samples showed pattern 0, and resembled more day 3 incubator controls with PBS.

Dose range finding (DRF), N = 36

Conventional cigarettes: DRF, N = 18

Nicotine concentration: mg/l

Both the 0 (Sham) and 3.11 exposed samples showed an appearance either matching or resembling the control with minor changes, pattern 1.
Both the 8.25 and 15.4 exposed samples showed subtle-mild changes, patterns 1 and 2.
Both the 89.1 and 201 exposed samples showed markedly abnomal appearance, patterns 4 and 5.

Test item: DRF, N = 18

Nicotine concentration: mg/l

Both the 0 (Sham): and 2.18 exposed samples showed an appearance either matching or resembling the control with minor changes, pattern 1.
The 5.84, 11.5, 79.2 and 147.2 exposed samples showed subtle-mild changes, patterns 1 and 2.

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5. Conclusions

Gingival 3D organotypic cell culture incubator control samples closely resembled human gingival tissue samples. They showed a mild increase in keratinisation over the duration of the study. The incubator control samples were taken as the base line from which changes deviating from this were taken as abnormal findings. Without knowledge of the exposure categories, assessing the blinded samples and controls, there appeared to be a progression of changes deviating from 'normal', from subtle loss of distinction of each layers to virtual loss of the layers and keratinisation (parakeratosis) extending down to the membrane, with associated cytological atypia, nuclear degenerative changes, and overall reduced maturation. The changes were split into six categories with 0 = control/normal and 5 = the most extreme change. Pattern 0 is regarded as normal for the purposes of the study. All other patterns are regarded as maladaptive.

The incubator control samples (all covered with PBS) were similar across all categories. With increasing days to 'harvest', the thickness of the stratum corneum tended to increase. In contrast, the thickness of the stratum granulosum (granular layer) somewhat diminished, perhaps reflecting the increased keratinisation. Apoptosis was greater at day 2 and diminished over increasing days. Mitoses were sparse and did not seem to particularly differ over time.

The sham samples covered by PBS most often showed changes similar to the incubator controls (pattern 0) or less often showed minor changes of subtle blurring of anatomical layers within the epithelium (pattern 1). The samples exposed to conventional cigarettes at a nicotine concentration of 49.4 mg/l showed pattern 4 changes, and those exposed to a nicotine concentration of 84.6 mg/l showed the most marked changes (pattern 5). In summary, the changes observed with both concentrations of nicotine were of reduced maturation, basal atypia, parakeratosis and, with the highest dosage, apparent loss of the basal layer.

In comparison, test item samples exposed to a nicotine concentration of 0 mg/l (Sham) showed either an appearance similar to the incubator controls, or only slight alteration (patterns 0-1). The samples exposed to a nicotine concentration of 14.4 mg/l showed increasing loss of distinction between the layers of the epithelium (patterns 1-2). The samples exposed to a nicotine concentration of 54.6 mg/l showed a range of changes from minor through to blurred layers and mild atrophy (patterns 1, 2 and focal changes of pattern 3), whereas samples exposed to a nicotine concentration of 100.4 mg/l showed blurring of the layers, from mild to more prominent atrophy and degenerative nuclear changes (pattern 3 and in one pattern 4). In summary, test item samples exposed to comparable doses of nicotine to those in conventional cigarettes, showed less pronounced changes, but there were still signs of injury. In particular, cigarette smoke-exposed samples (49.4 mg/l) showed more marked changes than samples exposed to the test item

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aerosol at only a slightly greater concentration of 54.6 mg/l. Samples exposed to conventional cigarette smoke at this concentration showed marked reduced maturation, parakeratosis, degenerative cell changes and basal atypia, whereas the test item exposes samples show changes as little as only subtle blurring of the anatomical layers through to moderate reduction in maturation with parakeratosis and some basal atypia. Additionally, samples exposed to the test item aerosol at a nicotine concentration of 100.4 mg/l in only one sample showed comparable changes (pattern 4, see section 4.4 above) to samples exposed to conventional cigarette smoke at a nicotine concentration of 49.4 mg/l, less than half the dosage. Some of the samples exposed to the test item at 14.4 mg/l and even at 54.6 mg/l still only showed an appearance similar to sham samples (pattern 1), but there were a range of patterns seen (see section 4.4 above).

The sham samples without PBS all in general displayed an appearance resembling the incubator controls (pattern 0), but had a thinner stratum corneum than shams with PBS. In summary, in PBS-covered samples the stratum corneum made up to 80% of the thickness of the sample, whereas in samples without PBS it was about 66%. The conventional cigarette smoke-exposed samples (nicotine concentration 49.4 mg/l) were similar to the same nicotine concentration with PBS, with slightly more hypergranulosis than seen with PBS for same degree of change. The test item aerosol-exposed sample (nicotine concentration 14.4 mg/l) also showed an appearance resembling the incubator controls (pattern 0), and resembled more day 3 incubator controls with PBS. The stratum corneum was slightly reduced in comparison. In summary, in sham samples, without PBS the thickness of the stratum corneum is reduced. In conventional cigarette smoke-exposed samples, there was little difference with or without PBS. In test item aerosol-exposed samples, there were less changes seen without PBS than seen with PBS, the reason for this is unclear. The numbers are small however, and the concentration of the smoke/aerosol low.

The changes observed in the DRF across the control samples were comparable to main phase controls. The sham samples across both cigarettes and test samples were comparable across the phases of the study (DRF to the main phases). The DRF, showed in both cigarettes and test items-treated cultures increasing changes with increasing dosage.
The samples exposed to conventional cigarette smoke at the highest nicotine concentrations showed prominent changes (patterns 4 and 5 as previously described), the changes comparable to the highest doses in the main phases. However, the test item samples never showed more than pattern 1 or 2 changes, even at doses higher than those tested in the main phases. Even the highest dosage of 147.2 mg/l showed only mild changes (pattern 1-2 as previously described), in the three samples. It is unclear why the alteration was less marked than seen in test items exposed samples in the main phases at a high, but lower dosage.

6. Disclaimer

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This is an exploratory non-GLP study which is compromised by relatively small the sample numbers across all groups.

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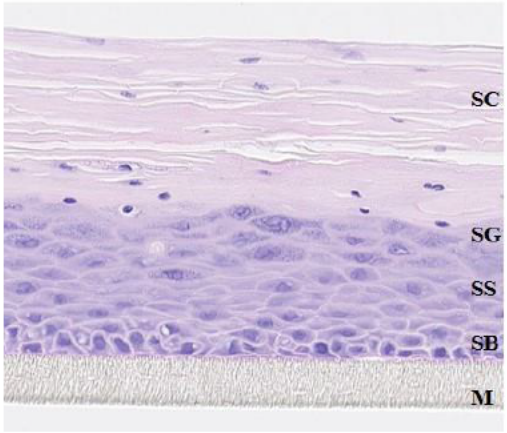
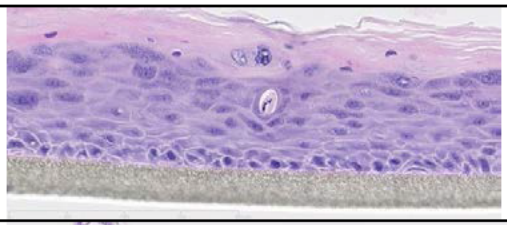
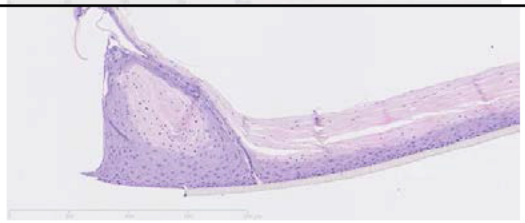
7. Appendix

7.1. Table 1

Finding	Explanation	Representative Picture of the Finding (gingival)
Assessment of layers	Measurement of cornified layer as a % of the whole. SC: Stratum corneum SG: Stratum granulosum (granular layer) SS: Stratum spinosum SB: Stratum basalis M: membrane <u>Layers are discrete</u>	
Apoptosis	Single cell degeneration	
Artefact at tissue border	Alterations found to be systematically present and deemed to be unlikely associated with the exposure. Uniformly present.	

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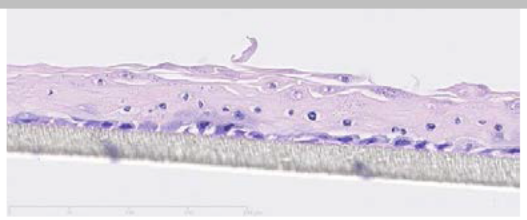
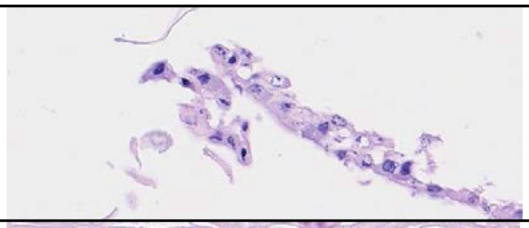
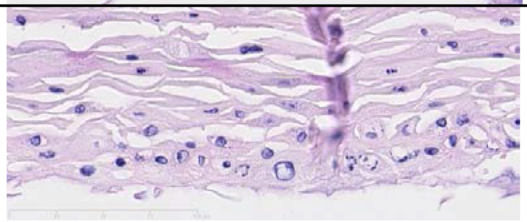
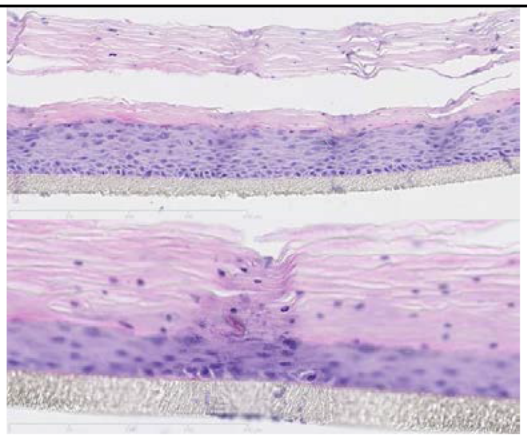
Atrophy	Thinning of non-keratinised portion of epithelium (lower layers) or whole epithelium	
Atypia	Nuclear irregularity, abnormal chromatin and increased nuclear:cytoplasmic ratio.	
Cell degeneration, incl apoptosis / karyorrhexis	Degeneration of cells seen often with fragmentation and vacuolation of cytoplasm and break up of nuclei.	
Corrugations	Parallel ridges or grooves in the culture perpendicular to the long axis. (lifting of tissue from slide and scanned in a different plane to adherent epithelium) A very common artefact. Occasionally the scan focussed on the smaller corrugations so much of the culture was blurred.	
Dyskeratosis	Abnormal single cell keratinisation	Not seen in this study

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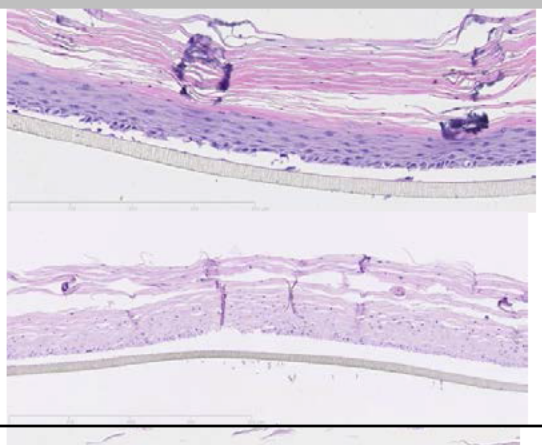
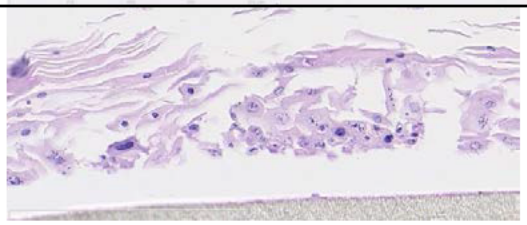
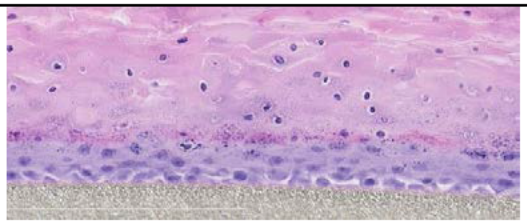
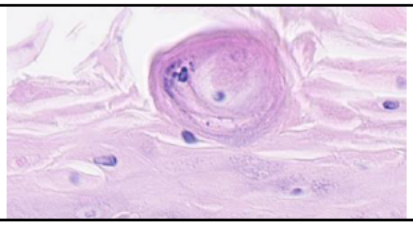
detachment from membrane	artefact (upper image) or as an abnormal finding due to reduced culture integrity in damaged epithelium (lower image)	
Epithelial disintegration and splits within epithelium (not within normal stratum corneum)	Loss of cell cohesion and fragmentation within damaged epithelium	
Hypergranulosis	Increased keratohyaline granules, included span within tissue and mainly increased coarseness	
Keratin pearls	Abnormal rounded keratinised structure with concentric layers of keratinisation.	
Mineralisation	Typical abnormal (dystrophic) calcification – mentioned in guidelines on oral mucosa assessment but not noted in this study	

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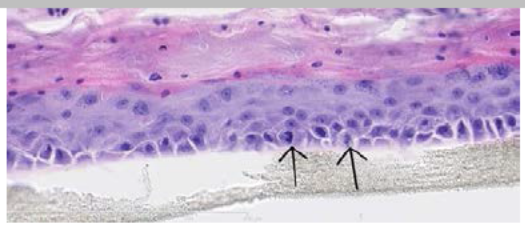
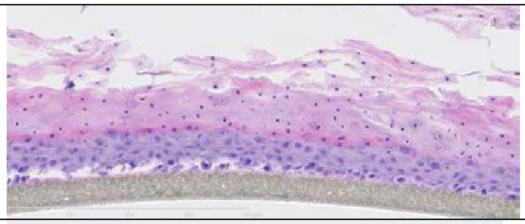
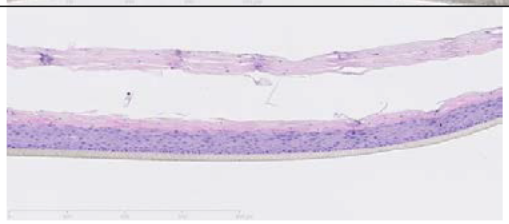
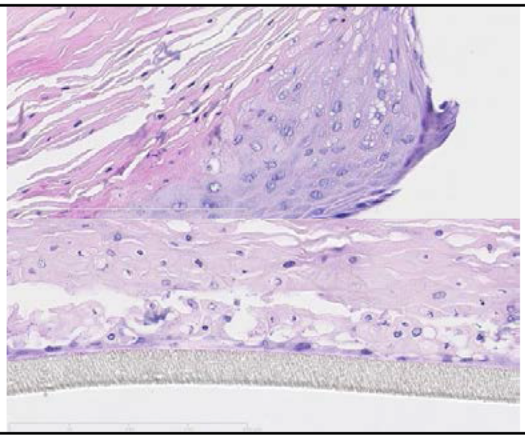
Mitoses	Present within basal layer in control epithelium and above basal layer in damaged / regenerating epithelium	
Parakeratosis	Excess persistence of nuclei within keratinised epithelium	
Split within stratum corneum (intracorneal split)	Separation of keratin layers, as one or more splits, more common when keratin is less compact – uniformly present	
Vacuolation	Nuclear clearing. Seen as an artefact at the edge (top image), and also as a finding within degenerative epithelium (bottom image).	

8. Reference

1. (reference: Histology for Pathologists, Chapter 15: Mouth, Nose, and Paranasal Sinuses. Stacey E. Mills. 2012 Wolters Kluwer)

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