Optimization of Seeding Density of Primary Human Gingival Fibroblasts for *in vitro* Toxicity Testing

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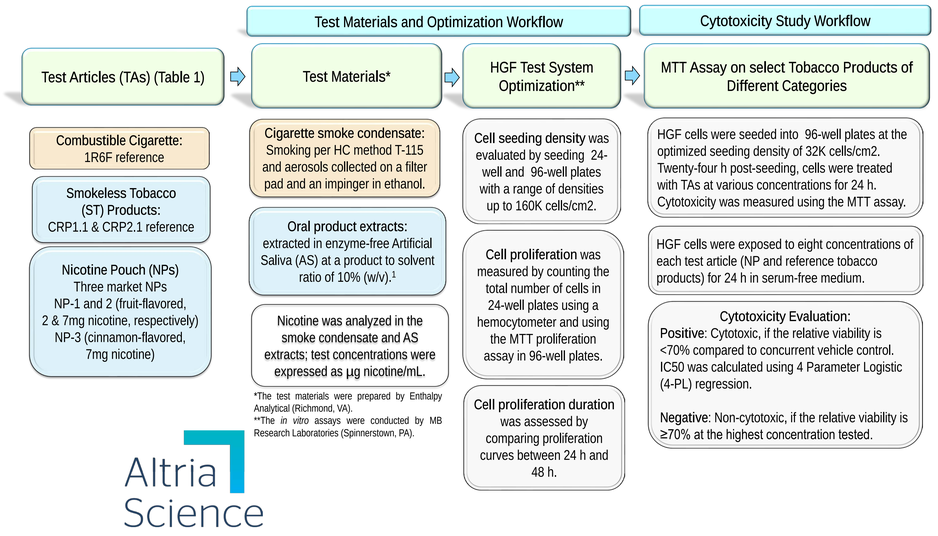
Abstract

Human gingival fibroblasts (HGFs), the predominant resident cells of the gingival connective tissue, are capable of self-renewal and secreting inflammatory molecules when stimulated and thus play a key role in the remodeling and disease development in periodontal tissues. Therefore, it is a physiologically relevant *in vitro* model for assessing the potential effects of tobacco and nicotine products on oral health (Figure 1). In this test system qualification study, we determined seeding densities of HGFs in 24- and 96-well tissue culture plates that would reach the target confluency of 70-80% after 24 or 48 h of culture to avoid the adverse effects from over confluency. HGFs were seeded at densities up to 160K cells/cm^2 per well and cell number was measured using a hemocytometer (in 24-well plates) and proliferation was measured using the MTT assay (in 96-well plates) after 24 and 48 h of culture. Cell number increased linearly at seeding densities up to 60K cells/cm^2 and plateaued at higher seeding densities 24 h after plating. Cell proliferation curves at both time points, as measured by the MTT assay, were similar and absorbance almost doubled at seeding densities up to 30K cells/cm^2. At seeding densities higher than 30K/cm^2, HGFs proliferated at a slower rate, resulting in a slower increase in MTT absorbance and lower absorbance at 48 h compared to 24 h; microscopic evaluation did not reveal obvious cell death, although cells appeared more aggregated in some areas of the wells. These findings suggest that HGFs may have undergone growth arrest possibly caused by contact inhibition at high seeding densities. Seeding densities in the range of 20K to 30K cells/cm^2 and treatment durations of 24 h are appropriate for acute toxicity testing.

Study Objectives

- Pre-study optimization of the test system (HGF)'s seeding density before in vitro toxicity testing of tobacco products
- Feasibility testing of the HGF *in vitro* model by measuring cytotoxicity of select tobacco products of different categories

Materials and Methods



Results

Figure 1*. Placement of Oral Nicotine Pouch (NP) in the mouth.
NPs are placed between the upper lip and gum during usage. Considering the direct contact of NPs with the gum, human gingival fibroblast (HGF) is a physiologically relevant *in vitro* model for assessing oral toxicity potential of NPs. *Created with BioRender.com

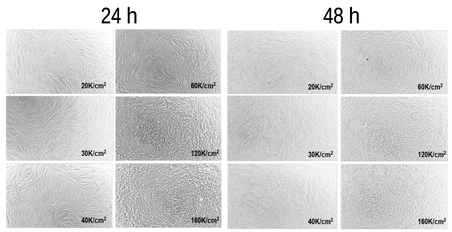


Figure 2. Microscopic Assessment on HGF Morphology.
Morphology of HGFs was evaluated using light microscopy 24 h and 48 h after seeding. Representative images are shown here. Confluency of cells increased with increasing proliferation time (24 h vs. 48 h). No obvious cell death (floating cells) was observed. However, cells appeared more aggregated in some areas for seeding density up to 60 K/cm^2.

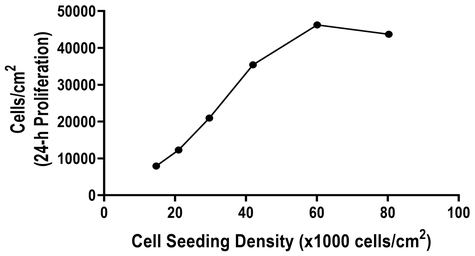


Figure 3. Preliminary Cell Count in a 24-well Plate.
Twenty-four hours after proliferation, cells were trypsinized and cell number counted using a hemocytometer. Cell number increased with increasing seeding densities.

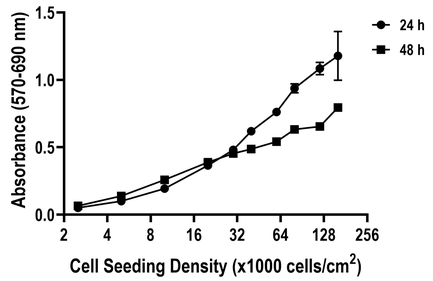


Figure 4. Proliferation of HGFs in 96-well Plate.
Proliferation of cells was measured using the MTT assay 24 h and 48 h after seeding. Proliferation of cells increased linearly (R^2 > 0.9) with increasing cell seeding densities at 24 h. Cell proliferation slowed down 48 h after cell seeding, especially at seeding densities higher than 30K/cm^2, compared to the 24-h time point. The proliferation curves at seeding densities lower than 30 K/cm^2 were similar between the 2 time points.

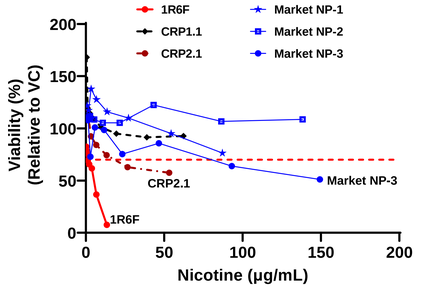


Figure 5. MTT Cytotoxicity assay
- Reference 1R6F cigarette was cytotoxic, showing a concentration-dependent decrease in viability (IC_{50} of approximately 6.76 µg/mL).
- CRP2.1 was also cytotoxic, with cell viability <70%, but its IC_{50} (67.5 ug/mL) was approximately 10-fold higher compared to 1R6F.
- The cinnamon-flavored Market NP-3 was also cytotoxic, but at higher exposure concentrations. Its IC_{50} (31.6 µg/mL) is approximately 5-fold higher compared to 1R6F.
- CRP1.1 and the other two (fruit-flavored) Market NPs were not cytotoxic (i.e., viability >70%), at all test concentrations.
- The approximate ranking of the cytotoxicity among the TAs is: 1R6F > Market NP-3 > CRP2.1 >> Market NP-1, Market NP-2.

Table 1. Summary of *in vitro* MTT Cytotoxicity of Tested Products

Test Products		Test Concentration (µg nicotine/mL)	IC_{50} (µg nicotine/mL)	IC50 Comparison to 1R6F Cigarettes
Reference Cigarette	1R6F	0.23-13.40	Cytotoxic (6.76)	(1)
ST Reference Products	CRP1.1 (reference snus)	1.21-62.30	Not cytotoxic	Negative
	CRP2.1 (reference moist snuff)	0.83-85.10	Cytotoxic (67.46)	~10-fold less cytotoxic
Market NP-1	Citrus, 2 mg	0.84-87.10	Not cytotoxic	Negative
Market NP-2	Dragon Fruit, 7 mg	1.34-138.30	Not cytotoxic	Negative
Market NP-3	Cinnamon, 7 mg	1.45-149.30	Cytotoxic (31.6)	~5-fold less cytotoxic

Conclusions

1. We established in vitro testing conditions, i.e., seeding density of ~30K cells/cm^2 and proliferation time of 24 h, appropriate for oral product toxicity study;
2. The in vitro MTT assay in HGF cells can differentiate the in vitro toxicity potential amongst different categories of tobacco products (e.g., combustible cigarette >> oral tobacco products), demonstrating the feasibility of HGF cells as an in vitro cell model for tobacco product testing.
3. Strengths and limitations

-- HGFs is a clinically relevant in vitro model for assessing the local effects of oral tobacco products;

-- Cytotoxicity is measured in HGFs proliferated under optimized seeding density and proliferation duration;

-- Separation of the response between different product categories is based on cytotoxicity. Additional endpoints reflecting other modes of action for NPs could provide further insights into the toxicity profiles of NPs.

References

1. Kumar, A., Doshi, U., Farcas, M., Zhang, M., Marzillier, J., DeGeorge, G., Lee, M. (2022). Mechanistic Toxicity Assessment of Oral Nicotine Pouches in Comparison to Combustible Cigarettes and Smokeless Tobacco Using Human Gingival Fibroblasts. 75th TCRC.

2. Khazaee, M., Cao, X., Farcas, M.T., Doshi, U., Zhang, M., Prepelitskaya, Y.V., Lee, K.M. Comparative Toxicity Evaluation of Oral Nicotine Pouch Products to Combustible Cigarettes and Oral Tobacco Comparator Products in Human Gingival Fibroblasts Using In Vitro Mechanistic Assays. Society of Toxicology 62nd Annual Meeting, March 19-23, Nashville, TN