Developing a Repeated Exposure Regimen Using a Buccal Epithelium-Fibroblast Tissue Model

Cao, X.[1], Molignano, J.[2], Farcas, M.T.[1], Oldach, J.[2], Gutierrez, M.M.[1]
[1]Altria Client Services LLC, 601 E. Jackson St., Richmond, VA 23219, USA
[2]MatTek Corporation, 200 Homer Ave, Ashland, MA 01721
Society of Toxicology 65th Annual Meeting and ToxExpo
March 22-25, 2026
Poster Board number: G548
Abstract ID#: 3405

Background and Purpose

Oral tobacco products (OTPs) are considered potentially reduced-risk alternatives to traditional combustible cigarettes within the tobacco harm reduction paradigm. However, their mechanistic toxicity profiles, particularly local effects on oral tissues, remain insufficiently characterized. Current mechanistic assays have largely focused on single-exposure designs, which are limited in their ability to capture the potential cumulative biological effects associated with repeated use, a pattern more representative of real-world consumer use behavior. Therefore, evaluating repeated exposure is an essential step toward addressing this knowledge gap and achieving a better understanding of oral tissue responses relevant to human outcomes. In this preliminary study, we established a feasible approach for conducting up to five repeated exposures using a human buccal epithelium-fibroblast co-culture tissue model (ORL-300 FT, MatTek Corporation, MA, USA).

Materials and Methods

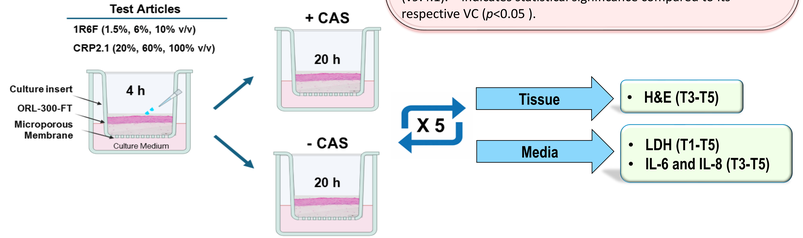
Test Article Preparation[a]: Kentucky 1R6F reference cigarettes were smoked according to the ISO 20778 (2018) smoking regimen. Condensates were collected from a total of 42 cigarettes across 3 smoking runs and extracted into 30 mL of USP-grade ethanol.
CRP2.1 moist snuff smokeless tobacco was extracted at a concentration of 30% (w/v) in complete artificial saliva (CAS) as described previously (Cao et al. 2025).

[a] The test materials were prepared by McKinney Specialty Labs (Richmond, VA).
[b] The in vitro assays were conducted by MatTek Corporation (Ashland, MA).

LDH[b]: LDH release was measured from treatment 1 (T1) to treatment 5 (T5) using an LDH Cytotoxicity Detection kit (Sigma-Aldrich). Cell viability was calculated using the following formula:

%viability vs. VC = 100 – [Abs(X) – Abs (VC)] / [Abs(PC) – Abs (VC)]x100
VC: vehicle control; PC: positive control (1% Triton X-100)

IL-6 & IL-8 ELISAb: IL-6 & IL-8 secretions were quantified at T3, T4, and T5 using commercially available ELISA kits (R&D Systems).
H&E Staining: Tissues were fixed in 10% formalin, cut in half, embedded in paraffin, sectioned at 5 µm. Section were stained with hematoxylin and eosin (H&E).
Statistical Analysis performed using one-way ANOVA in GraphPad (v9.4.1). * indicates statistical significance compared to its respective VC (p<0.05).



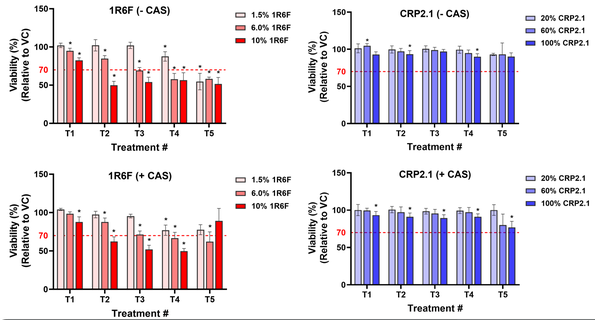
Strengths and Limitations

Strengths:
- A repeated-exposure regimen was employed to better reflect cumulative effects of OTPs.
- A human buccal epithelium-fibroblast co-culture model was used, enabling assessment of both epithelial and dermal responses relevant to oral tissue toxicity.
- The epithelial cells and fibroblasts used in this study were derived from the same donor, thereby minimizing inter-donor variability.
- Integration of cytotoxicity, cytokine secretion, and histopathology endpoints provided a multidimensional evaluation of tissue responses.
- The role of CAS as a modifying factor was systematically examined across exposure conditions.

Limitations:
- Most assays were conducted with three technical replicates, which may have limited their statistical robustness and sensitivity to subtle effects.
- Due to intrinsic limitations of the ORL-300-FT tissue model, repeated exposure was restricted to five consecutive days to avoid excessive tissue cornification, which may limit the ability to capture real-world chronic use scenarios.

Results

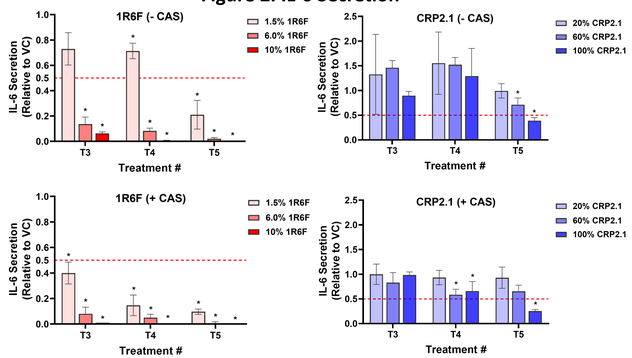
Figure 1. Cytotoxicity by LDH Assay



- Repeated exposure to 1R6F condensate resulted in a time- and concentration-dependent decrease in viability.
- CRP2.1 extract only marginally reduced viability, with the maximum observed decrease being less than 25%.
- No appreciable differences in viability reduction were observed between conditions with or without CAS.

VC was set to 100%

Figure 2. IL-6 Secretion



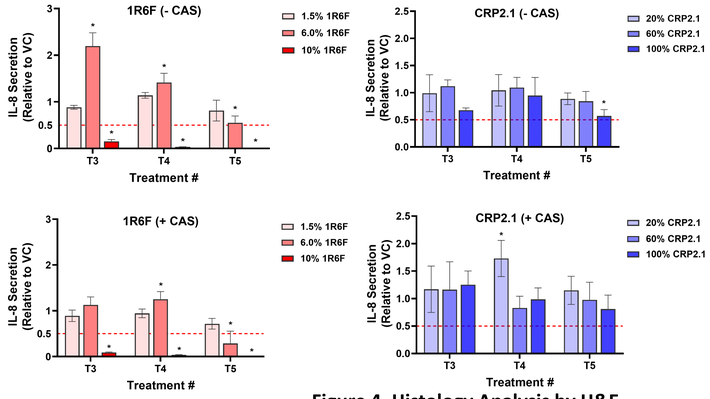
Effects of 1R6F
- Repeated exposure to 1R6F condensate markedly reduced IL-6 secretion in a concentration- and time-dependent manner.
- IL-6 secretion was completely inhibited at 6.0% and 10% following five consecutive treatments.
- The presence of CAS further enhanced the suppression of IL-6 release caused by 1R6F condensate.
- Given the cytotoxicity exhibited by 10% 1R6F at multiple timepoints and 6% 1R6F under some conditions, IL-6 reduction may be attributable to cytotoxicity.

Effects of CRP2.1
- CRP2.1 exhibited minimal effects on IL-6 secretion at T3 both in the presence and absence of CAS.
- CAS enhanced the reduction in IL-6 levels across all three concentrations at T4.
- CRP2.1 caused concentration-dependent decreases in IL-6 secretion, regardless of CAS presence.

VC was set to 1.

References
Cao X, Farcas MT, Prepelitskaya YV, Ritzenthaler A, Molignano J, Oldach J, Gutierrez MM. (2025). Comparative Evaluation of Artificial Saliva and Complete Artificial Saliva as Solvent Vehicles for *in vitro* Toxicity Testing of Oral Tobacco Products. Front Toxicol. 7:1657073.

Figure 3. IL-8 Secretion

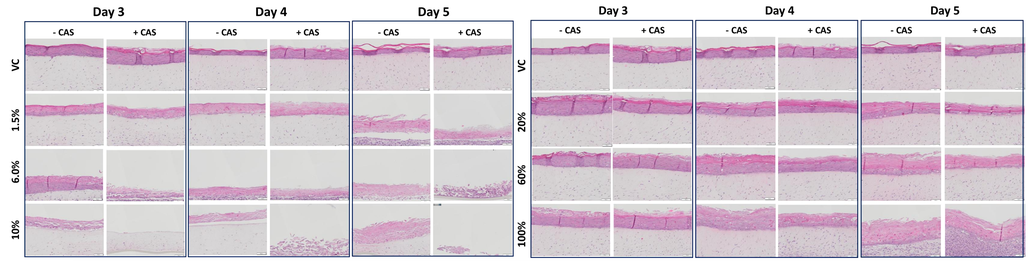


Effects of 1R6F
- 10% 1R6F condensate decreased IL-8 secretion at all three timepoints.
- IL-8 secretion was initially increased at 6.0% and gradually decreased with increasing number of treatments.
- The effects of 1R6F on IL-8 secretion were independent of CAS conditions.
- Given the cytotoxicity exhibited by 10% 1R6F at multiple timepoints and 6% 1R6F under some conditions, IL-8 reduction may be attributable to cytotoxicity.

Effects of CRP2.1
- The effects of CRP2.1 on IL-8 secretion were overall less pronounced.
- Reductions in IL-8 levels did not exceed 50% regardless of CAS conditions.

VC was set to 1.

Figure 4. Histology Analysis by H&E



- H&E histological evaluation demonstrated epithelial and dermal toxicity following exposure to both 1R6F condensate and CRP2.1 extract.
- Higher concentrations induced early signs of tissue alterations, demonstrated by tissue disintegration as early as T3, while lower concentrations resulted in moderate toxicity by T5.
- 1R6F elicited more severe and earlier tissue damage than CRP2.1, with frequent dermal involvement.
- Post exposure to CAS consistently exacerbated tissue injury, accelerating epithelial loss, dermal alterations, and overall tissue compromise.
- CAS-exposed control tissues remained largely morphologically normal, with only mild cornification (example indicated by arrow) observed overtime.

Conclusions

- This preliminary study demonstrates the feasibility of a repeated-exposure regimen using a human buccal epithelium–fibroblast co-culture model to assess local toxicity associated with tobacco products.
- The presence of CAS during the daily recovery phase exerted significant, time-dependent effects on IL-6 secretion and histopathological outcomes.
- These findings underscore the importance of accounting for CAS-related effects under extended exposure conditions.
- The results highlight the need to develop standardized, reproducible protocols capable of capturing potential cumulative biological responses over prolonged timeframes.