Evaluation of a Reconstructed Human Oral Buccal Tissue Model as a Testing Platform for Determining the Oral Irritation Potential of Tobacco Products

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ABSTRACT

There is an increasing need by the tobacco industry to evaluate the irritation and inflammation potential of tobacco products to support product development goals, for sound product stewardship, and likely regulatory safety tests. The use of *in vitro* human cell and tissue-based test methods to replace *in vivo* animal models addresses the need for more human-relevant predictive tools, and is consistent with many corporate animal welfare policies. Although monolayer cell-based cytotoxicity and cytokine expression assays have been used, three-dimensional tissue constructs provide distinct advantages since tissue exposures and pharmacokinetics more closely resemble the *in vivo* events. In this study, we evaluated a reconstructed human oral buccal model for determining the oral irritation of oral tobacco products. A dilution series of tobacco extracts were applied topically onto the EpiOral™ reconstructed human oral buccal model (Cat no. ORL-200) (MatTek Corporation, Ashland, MA) for various exposure times (ranging from 2 to 16 hours) to estimate oral irritation potential based upon reduction in cell viability and the synthesis/release of the inflammatory mediators IL-1α and IL-8. We determined tobacco extract concentration-related increases in cytotoxicity for the highest tobacco extract concentrations. We also found that increases in IL-1α release (up to 19-fold) generally correlated with the cytotoxicity increases. Exposure time-related increases in IL-8 release were generally observed in tissues treated with the three lower tobacco extract concentrations where relative viabilities were sufficiently high to allow for secondary cytokine production, but at the highest tobacco extract concentrations IL-8 release were below control levels where cytotoxic effects inhibited the cells' ability to synthesize proteins. These results demonstrate the utility of reconstructed human epithelial models for evaluating the irritation potential of tobacco products. To expand upon this utility, we propose to apply these general methods for determining cytotoxicity and inflammatory cytokine profiles to evaluating inflammation responses in reconstructed human airway tissue models exposed to combustible tobacco product extracts, particulate matter, and whole smoke.

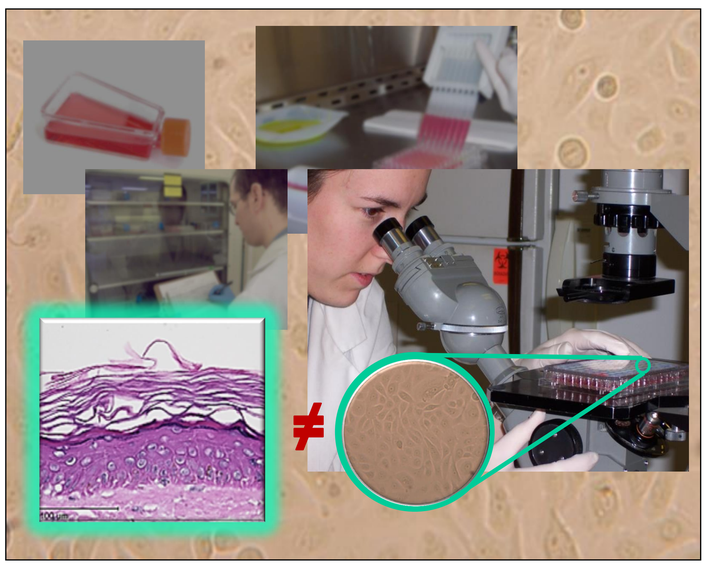
INTRODUCTION

Monolayer Cell Systems are:

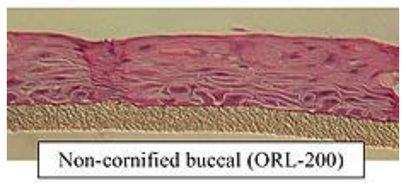
- Generally easy to conduct – cell lines
- Generally quite rapid to execute
- Evaluate individual chemicals (ingredients) rather than formulations
- Support HTP using robotics
- Machine scored endpoints
- Hazard oriented

However limitations include:

- Aqueous insoluble materials
- Dilution effects which mask toxicity of the neat material (e.g. ethanol)
- Buffering effects of the vehicle
- Pharmacokinetics poorly modeled
- No tissue barrier function modeled



Reconstructed Human Oral Buccal Tissue Model - MatTek Corp. EpiOral™ Tissue Model



General model characteristics:

- Stratified viable epithelial cells of human oral buccal origin
- Cultured at air-liquid interface
- *In vivo*-like barrier functions modeled
- Allow topical exposures of undiluted chemicals and formulations

MATERIALS & METHODS

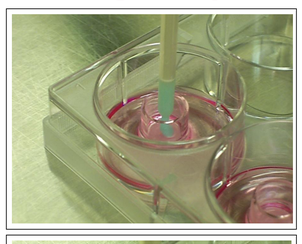
Tissue Receipt and Preparation

Reconstructed human tissue models and reagents are typically shipped refrigerated and stored at 2-8°C

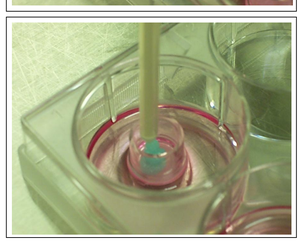
Tissues are transferred to 6-well plates that contain fresh assay medium

Tissues are incubated at 37°C, 5% CO_2, 90+% humidity (standard conditions)

Dosing – Topical Exposures



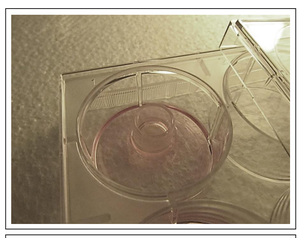
Oral tobacco products, product extracts or oral hygiene products are applied directly onto the oral tissue model surface



Tissues are incubated at standard conditions for a range of exposure times from 1 to 16 hours

Media supernatants are collected at the end of exposure for cytokine analyses by ELISA

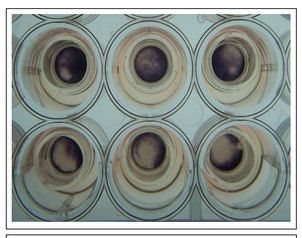
Rinsing of Treatments



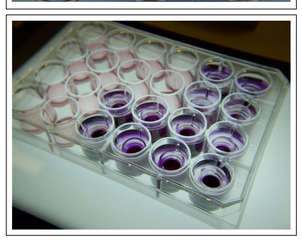
At the end of the exposure, test material is rinsed from the tissues with Dulbecco's phosphate buffered saline (D–PBS)

Thorough removal of test material is necessary to prevent prolonged exposure and over-predictions

Viability Assessment - MTT Reduction



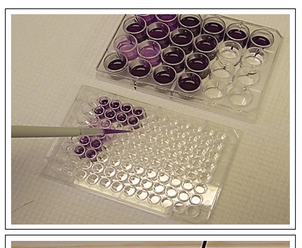
Individual tissues are incubated in unreduced MTT solution for 3 hours



Viable tissues convert MTT to a dark purple reduced form

The MTT is extracted from the tissues in isopropanol at room temperature for 2 hours

Viability Assessment – Quantification of Reduced MTT

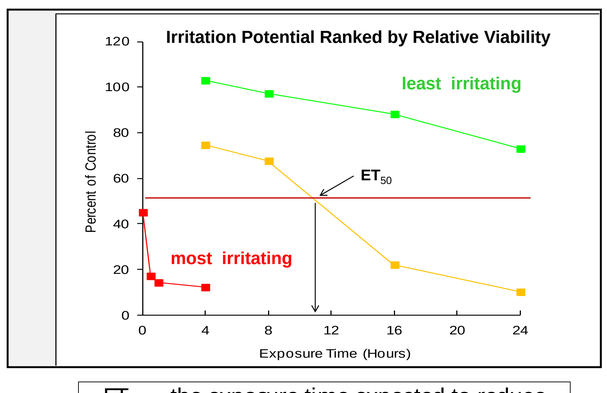


The optical density of extracted MTT is measured at 550 nm (OD_{550})

OD_{550} values are used to calculate relative viability values

Viability is presented relative to negative control tissue values

$$\% \text{ of Control} = \frac{\text{Test Material } OD_{550}}{\text{Negative Control } OD_{550}}$$



ET_{50} the exposure time expected to reduce viability to 50% of controls

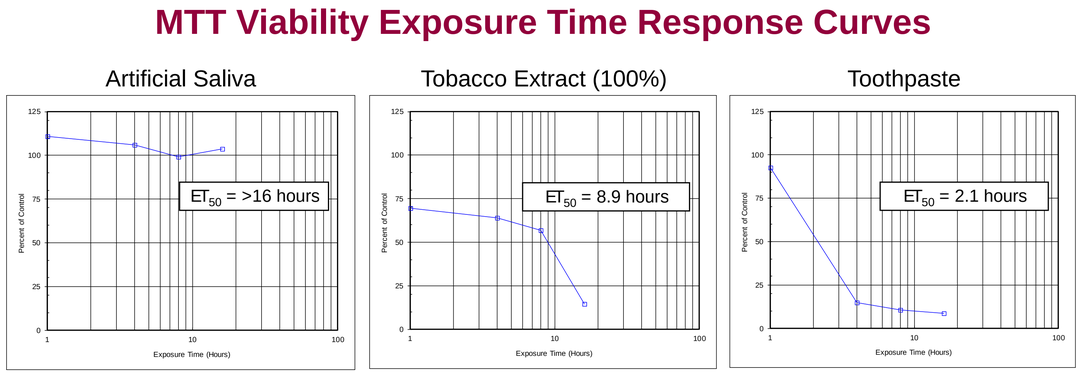
RESULTS

Exposure Time Range Finding Results

Test Product Designation	ET_{50} (hours)[1]	pH
Artificial Saliva	> 16	7.0
Tobacco Extract (100%)	8.9	discolored pH paper
Tobacco Extract (50%)	> 16	discolored pH paper
Mouthwash	9.6	5.0
Toothpaste	2.1	5.5

[1] ET_{50} is the exposure time expected to reduce viability to 50% of controls

MTT Viability Exposure Time Response Curves



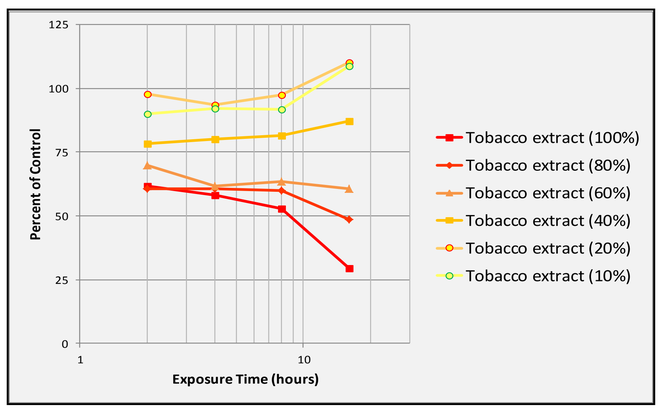
Two representative concentrations of tobacco extracts were initially tested in an exposure time range finding test. A commercial mouthwash and toothpaste were tested in parallel, for comparison. Based upon the exposure time responses obtained in the exposure time range finding test, relevant exposure times were selected for a series of tobacco extracts for the Definitive Assay. Duplicate tissues were treated in each treatment group. MTT viability and cytokine expression endpoints were determined in the Definitive Assay.

Definitive Assay Results

Test Product Designation	ET_{50} (hours)	Test Product Designation	ET_{50} (hours)
Artificial Saliva	> 16	Tobacco extract (100%)	8.7
Mouthwash	7.5	Tobacco extract (80%)	14.7
Toothpaste	2.9	Tobacco extract (60%)	> 16
		Tobacco extract (40%)	> 16
		Tobacco extract (20%)	> 16
1% Triton X-100 (assay control)	1.02 ✔	Tobacco extract (10%)	> 16

✔ The results of the positive control fell within the acceptable range established for the test system

MTT Viability Exposure Time Response Curves

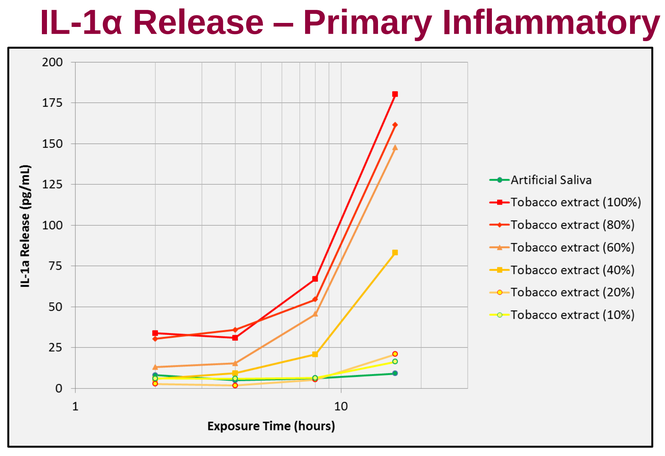


Tobacco extract concentration-related increases in cytotoxicity relative to time-matched Artificial Saliva controls

The lowest viability values were for the **highest** tobacco extract concentrations (100% and 80%)

Hyper MTT reduction at the two lowest tobacco extract concentrations (10% and 20%) suggest stress-related hormesis

IL-1α Release – Primary Inflammatory Cytokine

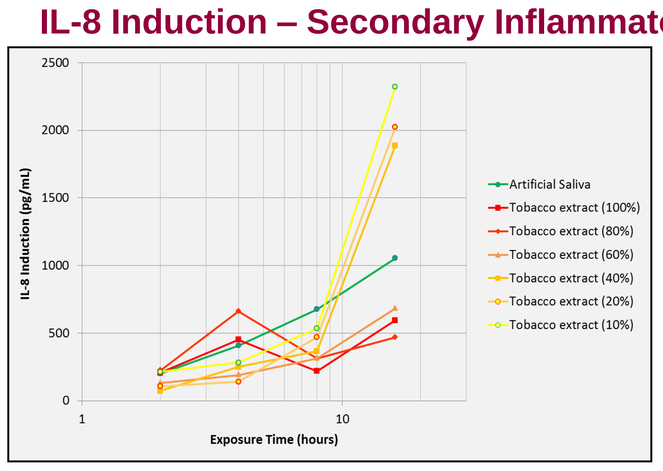


Tobacco extract concentration-related increases in IL-1α release in tobacco extract treated tissues, and time-matched Artificial Saliva

The highest IL-1α release values were for the **highest** tobacco extract concentrations (100%, 80%, and 60%)

Demonstrates the impact of cytotoxicity on cell membrane integrity and release of IL-1α

IL-8 Induction – Secondary Inflammatory Cytokine



Tobacco extract concentration-related impacts on IL-8 induction in tobacco extract treated tissues, and time-matched Artificial Saliva

IL-8 induction was observed at the **least** cytotoxic tobacco extract concentrations (10%, 20% and 40%), but was shut down at the higher cytotoxic concentrations

Demonstrates the inflammatory activity of tobacco extracts on oral tissues, and the adverse impact of cytotoxicity on IL-8 induction

CONCLUSIONS

Reconstructed human oral tissue models provide mechanistic evidence of the direct cytotoxic as well as inflammatory effects of tobacco extracts and oral tobacco products

- Cytotoxicity as a primary measure of irritation determined by the MTT viability endpoint
- Inflammatory cytokine expression profiles show the ability to respond to irritants by
  - release of the constitutively expressed primary cytokine IL-1α
  - induction and synthesis of the secondary inflammatory cytokine IL-8

We propose applying these endpoints to evaluate irritation and inflammation responses in other reconstructed human tissue models (e.g., in human airway tissue models exposed to tobacco product smoke extracts, particulate matter, or whole smoke)

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