In vitro Assessment of Artificial Saliva and Complete Artificial Saliva In Oral In Vitro Models

Cao, X.[1], Farcas, M.T.[1], Prepelitskaya, Y.V.[1], Molignano, J.[2], 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 64th Annual Meeting and ToxExpo
March 16-20, 2025
Poster Board number: G361
Abstract ID#: 3236

Background and Purpose

Oral nicotine pouches (NPs) are tobacco-leaf-free oral nicotine products that are considered to be potential reduced-risk alternatives to tobacco-based products as part of a tobacco harm reduction paradigm. However, the toxicity profiles of these relatively new NPs, especially their local (oral) toxicity, are not well understood. Non-clinical in vitro studies have been conducted, including standardized Organisation for Economic Co-operation and Development (OECD) genotoxicity tests and non-standardized mechanistic studies, to evaluate the toxicological characteristics of extracts from these products.

Unlike combustible cigarettes, no standardized methods are available for extracting smokeless tobacco products, including NPs, for in vitro testing. For example, various solvents, such as phosphate-buffered saline (PBS), culture medium, DMSO, enzyme-free artificial saliva (AS), and complete artificial saliva (CAS), have been used for extracting orally used products, including tobacco products. Among these solvents, CAS is a saliva substitute closely resembling human saliva with most key components, including enzymes. Therefore, it is presumed to provide greater biological relevance compared to other solvents. To determine whether CAS is a more suitable alternative than AS for mechanistic testing, we conducted a study to compare the extraction efficiency and a range of biological effects elicited by AS and CAS.

Materials and Methods

Test Materials*

Artificial Saliva:
- Enzyme-free Artificial Saliva (AS)
- Complete Artificial Saliva (CAS)

Extraction Efficiency Test:
- Extraction of CRP1.1 in AS and CAS at 10%, 20%, and 30% (w/v)
- Concentrations of nicotine and TSNAs (e.g., NAB, NAT, NNK, and NNN) were quantified as measures for extraction efficiency.

In Vitro Test Systems

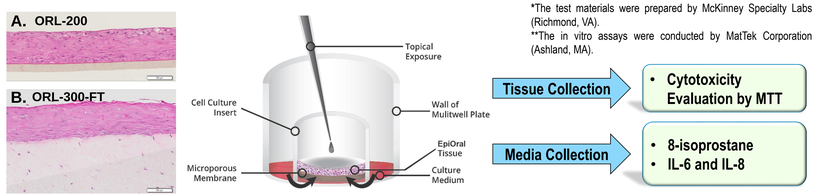
Two-Dimensional (2D) Monolayer Cells:
- Normal Human Gingival Fibroblasts (NHGFs)
- Normal Human Oral Epithelial Cells (NHOEs)

Organotypic Oral Tissue Models:
- A) EpiOral™ Buccal Tissue Model (ORL-200)
- B) EpiOral™ -Full Thickness (FT) Buccal-Gingival Tissue Model (ORL-300-FT)

In vitro Mechanistic Assays**

Outcomes:
Cytotoxicity assessed by MTT
Oxidative Stress assessed by 8-Isoprostane ELISA
Inflammatory Responses assessed by IL-6 and IL-8 ELISA
Statistical Analysis performed using one-way ANOVA in GraphPad (v10.4.1). $p<0.05$ indicates statistical significance.

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



Tissue Collection → Cytotoxicity Evaluation by MTT

Media Collection → 8-isoprostane; IL-6 and IL-8

Strengths and Limitations

Strengths:
- Conventionally, vehicle control effects are mainly assessed by employing cytotoxicity assays. The inclusion of additional endpoints allows a better understanding of their biological effects.
- Epithelial cells and fibroblasts utilized in this study are also used for generating ORL-300-FT tissue models, avoiding inter-donor variability.
- Given the intrinsic variation of the 3D tissue models, we used n=4 in an effort to increase the power of the assay and ensuring reliable and meaningful results.

Limitations:
- We selected TEER-GLC buffer as the experimental vehicle control and dilution buffer. Therefore, composition of the AS/CAS treatment solutions is not completely comparable with the experimental vehicle control.
- Integration of additional cytokine assays may benefit a full understanding of the effects of these artificial saliva on inflammation responses[1].

Altria Science

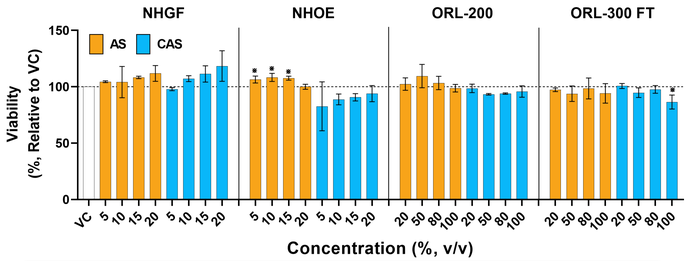
Results

Table 1. Extraction efficiency testing: Chemical analysis of select constituents of CRP1.1 extract in AS and CAS (Data is expressed as mean [SD]).

[Extract] (%w/v)		Nicotine (mg/mL)		TSNAs[a] (ng/mL)							
				NAB		NAT		NNK		NNN	
		Conc.	% Recov.	Conc.	% Recov. based on reference value[b]	Conc.	% Recov. based on reference value[b]	Conc.	% Recov. based on reference value[b]	Conc.	% Recov. based on reference value[b]
AS Extract	10%	0.652 [0.005]	83.8 [0.7]	0.770 [0.034]	85.3 [3.7]	12.10 [0.17]	86.2 [1.1]	4.81 [0.03]	92.2 [0.5]	17.30 [0.50]	90.7 [2.6]
	20%	1.203 [0.006]	77.0 [0.4]	1.137 [0.090]	62.7 [4.9]	18.47 [1.82]	65.7 [6.4]	7.84 [0.90]	75.0 [8.6]	29.27 [2.57]	76.7 [6.7]
	30%	1.503 [0.006]	64.6 [0.3]	1.267 [0.090]	46.9 [3.2]	19.73 [0.85]	47.0 [2.1]	8.38 [0.46]	53.6 [2.9]	32.30 [1.71]	56.7 [3.0]
CAS Extract	10%	0.632 [0.003]	81.5 [0.3]	0.513 [0.062]	56.9 [6.9]	8.35 [0.30]	59.5 [2.1]	3.26 [0.17]	62.6 [3.4]	12.70 [1.08]	66.8 [5.5]
	20%	1.127 [0.015]	72.3 [0.8]	0.977 [0.181]	54.0 [10.0]	14.53 [2.14]	51.6 [7.6]	6.50 [1.23]	62.2 [11.7]	24.53 [3.53]	64.3 [9.2]
	30%	1.510 [0.020]	64.8 [0.8]	1.297 [0.015]	47.9 [0.6]	18.50 [1.11]	44.0 [2.7]	8.42 [0.90]	53.9 [5.8]	33.97 [3.12]	59.5 [5.4]

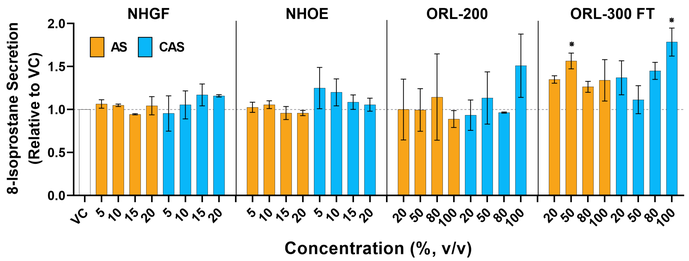
[a]TSNA: tobacco-specific nitrosamine; NAB: N-nitrosoanabasine; NAT: N-nitrosoanatabine; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN: N-nitrosonornicotine
[b]% calculated from mean value reported in the CORESTA 2017 study[2].

Figure 1. Viability by MTT



- Neither AS nor CAS induced a marked decrease in cytotoxicity at any test concentration in the four in vitro oral models.
- Although responses by AS at 5, 10, and 15% (v/v) in NHOE and CAS at 100% in ORL-300-FT were statistically significant, the changes were all below 15%.

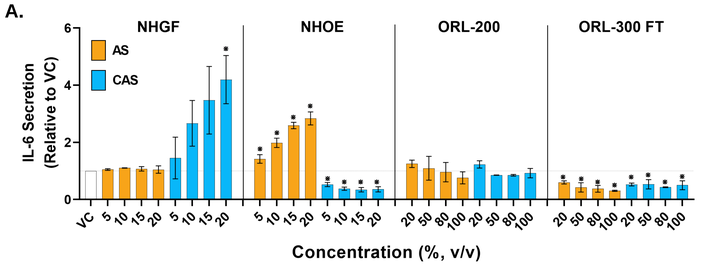
Figure 2. Oxidative Stress by 8-Isoprostane Secretion



- Neither AS nor CAS altered secretion of 8-isoprostane at all test concentrations in NHGF, NHOE, and ORL-200.
- 50% AS increased 8-isoprostane secretion by ~50% in ORL-300-FT. However, considering the lack of dose-response, such an increase may be due to intrinsic variations of the tissue model.
- 100% CAS induced 8-isoprostane secretion by ~1.8-fold with statistical significance in ORL-300-FT.

Figure 3. Inflammatory Responses (Secretion of IL-6 [A] and IL-8 [B] cytokine)

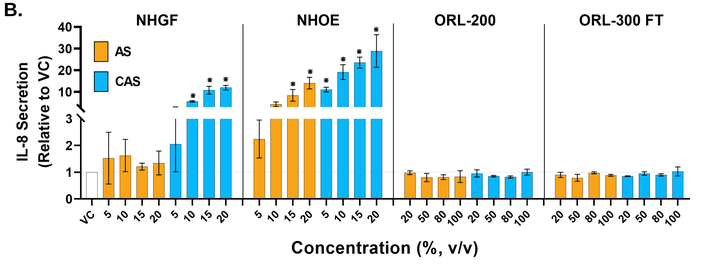
A.



In the 2D cells:
- AS dose-dependently induced IL-6 secretion in NHOE, but not in NHGF cells.
- CAS increased IL-6 secretion in NHGF cells but decreased IL-6 secretion in NHOE cells, indicating a reduced inflammation baseline level in epithelial cells.

In the 3D models:
- Neither AS nor CAS altered IL-6 secretion in ORL-200.
- Both AS and CAS reduced IL-6 secretion in ORL-300-FT, suggesting a reduced inflammation state.

B.



In the 2D cells:
- AS induced IL-8 secretion in NHOE cells, but not in NHGF cells.
- CAS dose-dependently increased IL-8 secretion by ~12- to 29-fold in NHGF and NHOE cells, respectively.

In the 3D models:
- Neither AS nor CAS had any effect on IL-8 secretion in ORL-200 and ORL-300-FT.

Conclusions

- Results from the extraction efficiency study indicate that 30% w/v extract concentration in AS and CAS produced acceptable extraction efficiency based on the recovery of nicotine and TSNAs.
- Overall, examining responses other than cytotoxicity is essential for a comprehensive evaluation of the baseline biological effects of vehicles used in in vitro mechanistic testing, as it provides a more comprehensive assessment of the vehicle's impact on cellular responses, enhances mechanistic insights, and improves the interpretation of experimental results.
- Based on our observations, CAS and ORL-300-FT were selected as the test systems for future testing of oral tobacco products due to their marginal vehicle control effect for most endpoints and their overall greater biological relevance.

References
1. Malpass, G.E., Arimilli, S., Prasad, G.L., Howlett, A.C. (2013). Complete Artificial Saliva Alters Expression of Proinflammatory Cytokines in Human Dermal Fibroblasts. Toxicological Sciences. 134:18-25.
2. Smokeless tobacco sub-group technical report: CORESTA reference products 2016 analysis. 2017. CORESTA. https://www.coresta.org/sites/default/files/technical_documents/main/STS-105-CTR_2016-CRP-AnalysisWG4_Jan2017.pdf