Development of a 3D Human Oral Tissue Model for Oral Permeation of **Smokeless Moist Snuff**

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Abstract

Tobacco product use is a risk factor in the development of oral cancer, although epidemiology studies show this risk is far less with smokeless tobacco product use than cigarette smoking. While smokeless tobacco contains harmful and potentially harmful constituents (HPHCs), the oral permeation of HPHCs is not completely understood. The goal of this study was to determine the suitability of a 3D human buccal oral tissue model, EpiOral™, to study this process by using an extract of CORESTA moist snuff smokeless tobacco reference (CRP2).

CRP2 extract was prepared in complete artificial saliva (CAS) with a ratio of 300 mg of CRP2 to 1 mL of CAS. sterile filtered and stored at -80°C until time of exposure with the stock extract or a 150 mg/mL dilution. CRP2 extract (150 or 300 mg/mL), CAS alone (vehicle control) or caffeine/CAS solution (0.15%: positive control) were applied to the apical side of EpiOral[™] tissues and permeation was measured for 3 hours with fresh receiver solution (basolateral compartment) replaced every 30 minutes. After the last time point, EpiOral[™] tissues were rinsed and cytotoxicity (via 3-(4,5-d-2-yl)-2,5diphenyltetrazolium bromide; MTT) was measured. Subsequently, tobacco-specific nitrosamines (TSNAs) and caffeine concentrations were measured in each receiver solution by liquid chromatography (LC).

Application of CRP2 extract (150 or 300 mg/mL), CAS alone or caffeine/CAS solution (0.15%) to the EpiOral™ tissues did not produce cytotoxicity as measured via MTT. Permeation over time (i.e., flux) was observed to achieve a linear steady state for each analyte measured over the 3 hours.

Collectively, these data suggest that the EpiOral[™] model could increase the understanding of the permeation of different tobacco constituents from moist snuff smokeless tobacco into oral tissue

References

1. Chou, C. C., and Hee, S. S. Bioassay-driven analysis of chewing tobacco extracts (1994). Environ. Toxicol. Chem. 13, 1177-86

2 Fowler KW Hill JA Bombick BR and Prasad G I Standardization of the preparation of smokeless tobacco extracts for assessment of biological effects Presentation: 64th Tobacco Science Research Conference, Hilton Head, SC, Oct 2-6, 2010

3. CORESTA Recommended Method 72. Determination of tobacco-specific nitrosamines in tobacco and tobacco products by LC-MS/MS. https://www.coresta.org/sites/default/files/technical_documents/main/CRM_72-Julv2017.pdf

4. Determination of caffeine in coffee products according to DIN 20481. https://www.agilent.com/cs/library/applications/5991-2851EN.pdf

Results



Figure 1: Schematic representation of experimental design. Receiver solution (PBS supplemented with 3.6 mg/mL of glucose) was collected in 30 minute intervals beginning at 30 minutes after application of the donor solution. Time points were conducted over a 3 hour period. Donor solutions were either 0.15% caffeine, 150 mg/mL or 300 mg/mL CRP2 (moist snuff reference product) in complete artificial saliva (CAS).



Figure 2. Cumulative TSNAs permeation in EpiOral[™] tissues. Permeation of N-nitrosonornicotine (NNN) (A), N'-nitrosoanatabine (NAT) (B), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (C), and Nnitrosoanabasine (D) was measured over 3 hours in EpiOral™ tissues exposed to CRP2 extract in CAS. Following each 30 minute time point, the receiver solutions were collected, stored at < 60°C and subsequently analyzed for TSNAs and cumulative permeation was plotted. Linear regression was performed and 95% confidence intervals are shown. Three different production lots were used over the course of the study (n=3 per time point).



Figure 3. Positive control assessment of caffeine cumulative permeation in EpiOral[™] tissues. Caffeine (0.15% in CAS) permeation was measured over 3 hours in EpiOral[™] tissues. Following each 30 minute time point. the receiver solutions were collected, stored at <-60°C and subsequently analyzed for caffeine and cumulative permeation was plotted. Linear regression was performed and 95% confidence intervals are shown. Three different production lots were used over the course of the study (n=3 per time point).

125 % Viability lative to CA⁴ 50 mg/ml CRP2 CRPL 0.15% caffein

Figure 4. EpiOral[™]tissue viability was assessed following the final permeation time point (3 hours). Viability was assessed by MTT and calculated relative to vehicle control (CAS). Three different production lots were used over the course of the study (n=3 per experiment).

Materials and Methods

3D Cell Model: Three production lots of EpiOral™ tissues were obtained from MatTek, Inc. These are comprised of normal, human-derived buccal cells cultured for 12 days to form a highly differentiated model. Tissues were maintained at the air liquid interface according to the manufacturer's quidelines.

Smokeless Tobacco Product: CRP2 was obtained from North Carolina State University. Extracts of CRP2 were prepared via extraction in Complete Artificial Saliva (CAS) (Chou and Hee, 1994) (1) on a weight to volume basis, as described previously (Fowler et al., 2010) (2),

Permeation Experiments: Extracts of CRP2 (300 or 150 mg/mL) in CAS were applied apically to EpiOral™ tissues. After apical application of CRP2, in 30 minute intervals, tissues (including CRP2 extract) were moved to a well containing PBS supplemented with 3.6 mg/mL glucose. Subsequently, each receiver solution was collected snap-frozen in liquid nitrogen and stored at $< 60^{\circ}$ C until analysis. This process was repeated for a total of 3 hours. After 3 hours. EpiOral™ tissues were rinsed with PBS supplemented with glucose, and cytotoxicity (via 3-(4,5-d-2-yl)-2,5diphenyltetrazolium bromide; MTT) was measured.

MTT Analysis: The MTT assay was performed according to the manufacturer's instructions (MatTek corporation, cat, # MTT-100). The reaction is quantified by measuring the absorbance of the purple formazan solution at 570 nm. Liquid Chromatography: The detection of tobacco-specific nitrosamines (TSNAs) and caffeine was as follows:

TSNAs: Analysis was conducted by liquid chromatography coupled with triple quadrupole mass spectroscopy (LC-MS/MS) using positive electrospray ionization (ESI) utilizing a Phenomenex Gemini C18 3.0 µm particle 2.0 x 150 mm column. Quantitation was achieved using external standard calibration. Method is based on CORESTA Recommended Method (CRM) 72 (3).

Caffeine: Analysis was conducted by liquid chromatography (LC) UV spectroscopy at 272 nm utilizing an Agilent Poroshell 120 EC-C18, 3.0 x 50 mm, 2.7 µm column. Quantitation was achieved using external standard calibration. The LC and quantitation methods are based on a previously published method by Agilent (4).

Conclusions

- Permeation of caffeine and TSNAs in CRP2 CAS extracts was $\frac{1}{2}$ observed and reached steady-state permeation (r²≥0.986)
- between 30 to 60 minutes (Figures 2 and 3). TSNAs and caffeine steady state permeation rates were consistent between the three production lots (Figures 2 and 3). TSNAs and caffeine steady state permeation rates were
- Steady-state flux (J_{ss}) of 150 mg/mL CRP2 TSNAs was approximately half of 300 mg/mL CRP2 TSNAs (Figure 2).
- ♦ Viability of EpiOralTM tissues was not significantly (p>0.05)
- Viability of EpiOral[™] tissues was not significantly (p>0.05) reduced when exposed to CRP2 or caffeine (Figure 4). The data suggest that the EpiOral[™] tissue model may be suitable to study permeation of TSNAs from smokeless tobacco products. ♦ The data suggest that the EpiOral[™] tissue model may be