Mechanistic Toxicity Assessment of Oral Nicotine Pouches in Comparison to Combustible Cigarettes and Smokeless Tobacco Using Human Gingival Fibroblasts

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Abstract

Oral Nicotine Pouches (NPs) constitute a growing alternative tobacco product segment in the United States. A rigorous toxicity assessment is integral to the scientific evaluation of potential health risks of NPs compared to other tobacco products. In this *in vitro* study, we tested eight commercially available comparator NPs of varying flavor and nicotine levels (extracted in artificial saliva [AS]) using primary human gingival fibroblasts (HGF) to evaluate mechanistic toxicity responses. We also included combustible reference cigarettes (3R4F and 1R6F; smoke condensate in ethanol) and oral tobacco products (reference moist smokeless tobacco CRP2.1, reference snus CRP1.1; both extracted in AS). HGF cells were exposed to eight concentrations (up to 149.3 µg/mL nicotine) of AS extracts for 24 hours. The cigarette smoke (CS) condensates elicited concentration dependent cytotoxicity (IC_{50}≤12.01 µg/mL nicotine); increased oxidative stress (elevated malondialdehyde [MDA], lowered levels of reduced glutathione (GSH) and its ratio with oxidized glutathione [GSH/GSSG]); and exacerbated inflammation (upregulated interleukin-8 [IL-8], matrix metalloproteinase-1 [MMP-1], and downregulated tissue inhibitor of metalloproteinase-1 [TIMP-1]). In comparison, tested market NPs exhibited varying responses for toxicity endpoints, with many products showing no changes under test conditions, although all NPs were substantially less toxic than CS. Reference oral tobacco products showed responses that mostly overlapped with the market NPs, except for some pronounced elevation in oxidative stress and inflammation markers (increased MDA, IL-8 and MMP-1) over NPs. In summary, mechanistic *in vitro* testing of inflammation and oxidative stress in primary HGF cells can help differentiate the toxicity potential among different tobacco product categories with all tested oral tobacco products exhibiting substantially lower toxicity than cigarettes.

Introduction

Underlying mechanisms, such as oxidative stress and inflammation have been identified as the two major contributors to smoking related chronic oral health conditions such as periodontal disease. Smoke-free nicotine products, such as NPs, are intended to be used via the oral route and are considered potential reduced-risk alternatives for adult smokers. However, since these products are relatively new, there is limited understanding of their effect on oral health. We assessed the effects of eight commercially available NPs on oral health using selected *in vitro* mechanistic endpoints (oxidative stress and inflammation) using a human-derived test system, primary human gingival fibroblasts (HGF). The *in vitro* toxicity responses of NPs were evaluated against the selected comparator products: combustible reference cigarettes and oral smokeless tobacco products.

Materials and Methods

IN VITRO TOXICOLOGICAL ASSESSMENT WORKFLOW

Products Tested → Test material preparation and Exposure* → Mechanistic Toxicological Aassessment → Criteria for Evaluating Assay Responses

- Combustible Tobacco Products (Cigarettes): 3R4F & 1R6F
- Smokeless Tobacco Products: CRP 1.1. & CRP 2.1
- Commercial Oral Nicotine Pouches: Eight Products

Cigarette smoke condensates were collected in ethanol, using ISO intense (ISO 20778) puffing regimen.

Oral product extracts were prepared in enzyme-free Artificial Saliva (AS) [1] using 10% product to volume ratio (w/v).
Nicotine concentrations in the extracts vary, depending on the amount of nicotine in the product and the number of pouches used to achieve the 10% w/v ratio.

HGF cells were exposed to up to eight concentrations of each test material for 24 hours

Cell Viability (MTT)

1. Oxidative Stress endpoints: Lipid Peroxidation (MDA), GSH, and GSH/GSSG
2. Inflammation and tissue damage markers: IL-8, TIMP-1, and MMP-1

Positive: Cytotoxic, if relative viability <70%.
Negative: Non-cytotoxic, if the relative viability of the test material was at. or higher than, 70%.

Outcomes:
Increase: ≥ 2-fold increase relative to the respective concurrent vehicle controls for following endpoint specific markers: MDA, IL-8, and MMP-1
Decrease: ≥ 2-fold decrease relative to the respective concurrent vehicle controls for following endpoint specific markers: GSH, GSH/GSSG, and TIMP-1
No Change:
If < 2-fold increase or no change relative to the respective concurrent vehicle controls for MDA, IL-8, and MMP-1
If < 2- fold decrease or no change, relative to the respective concurrent vehicle controls for GSH, GSH/GSSG , and TIMP-1

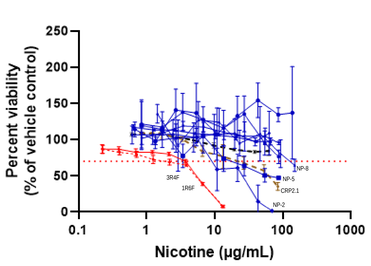
* The AS[1] and test material preparation was performed at Enthalpy Analytical, and the *in vitro* assays (exposure) were conducted at MB Research Labs.

Strengths & Limitations

Strengths: 1) We used a clinically relevant *in vitro* cell system (human primary cells relevant to oral exposure), supporting biological plausibility of reduced-risk potential of NPs in oral health. 2) Multiple markers were included for each endpoint to gain a better understanding of the overall effects of NPs and tobacco products (oral & combustible) on oxidative stress and inflammation.
Limitations: 1) We used nicotine to represent the extraction efficiency of test materials and assumed the extraction efficiency for other ingredients (e.g., flavors) to be complete but not confirmed analytically. 2) We selected market NPs with different nicotine levels, flavor varieties, and manufacturers; however, this may not be representative of the totality of available NPs and the use of the results for categorical evaluation warrants caution. 3) No guidelines exist in the literature for the conduct of mechanistic *in vitro* assays and primary human cells as test systems. There may be batch-to-batch variability as well as experimental variability within the same lot and caution is warranted when comparing data across multiple runs.

Results

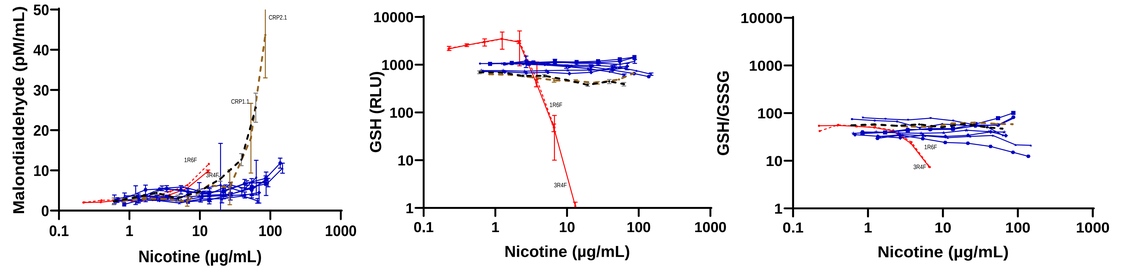
Figure 1. MTT Cytotoxicity Assay



The curves are plotted as mean responses ± SD from 3 replicates, normalized to nicotine in µg/mL

- Cigarette (3R4F and 1R6F) smoke condensates were cytotoxic (IC_{50} of < 12.01 µg/mL nicotine).
- While some oral tobacco products (CRP2.1, NP-2, NP-5, and NP-8) showed decrease in viability below 70% and were considered cytotoxic, other tested oral tobacco extracts were non-cytotoxic, even when tested at >10-fold higher nicotine concentrations.

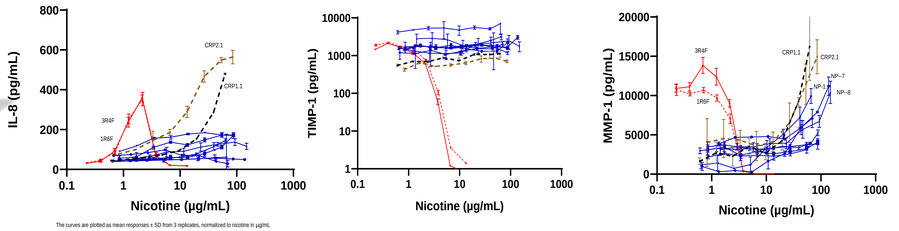
Figure 2. Oxidative Stress Endpoints



The curves are plotted as mean responses ± SD from 3 replicates, normalized to nicotine in µg/mL

- Cigarette (3R4F and 1R6F) smoke condensates exhibited a dose dependent increase in lipid peroxidation as shown by an increase of MDA (Left panel), and a decline in GSH (middle panel) or GSH/GSSG ratio (right panel), indicating pronounced oxidative stress.
- Among oral tobacco products, CRP2.1 and CRP1.1 showed notable increases in MDA at high concentrations compared to cigarettes. Most commercial NPs (NP-3, NP-4, NP-5, NP-6, NP-7, NP-8) also showed increases in MDA, but at higher concentrations, in comparison to cigarettes. Some commercial NPs (NP-3, NP-5, NP-6, NP-7, NP-8) also demonstrated a decrease in GSH/GSSG ratio at concentrations several fold higher than cigarettes, however these changes were slightly above 2-fold and were not accompanied by any decreases in GSH.

Figure 3. *In vitro* Inflammation and Tissue Damage Markers



The curves are plotted as mean responses ± SD from 3 replicates, normalized to nicotine in µg/mL

- Both (3R4F and 1R6F) cigarette smoke condensates induced a strong IL-8 (left panel) response (>16 fold) at low nicotine concentrations (<2.2 µg/ml nicotine) and then declined at higher concentrations correlating with decrease in cell viability. Cigarettes also elicited dose dependent decline in TIMP-1 (middle panel) and increase in MMP-1 (right panel), indicating pronounced inflammation.
- CRP1.1 and CRP2.1 also showed pronounced increases in IL-8 (≥ 7 folds) at high nicotine concentrations. Some commercial NPs (NP-3, NP-4, NP-5, NP-6, NP-8) also showed increases in IL-8 but to a lesser extent and only at high nicotine concentrations. For TIMP-1, all NPs (except for a slight decrease for NP-8) showed no decline under the tested condition. Oral tobacco products (CRP2.1, CRP1.1) did show notable increases in MMP-1 at high concentrations and commercial NPs (NP-1, NP-7, NP-8) also showed increases (> 2-fold) in MMP-1 only at high concentrations.

The responses with oral nicotine pouches varied depending on the product, but overall, the selected NPs had substantially lower toxicity (cytoxicity, oxidative stress and inflammation) than cigarettes and a comparable or lower toxicity to smokeless tobacco products, supporting their reduced-risk potential.

Table 1. Product Information, Figure Legends, and Summary of Mechanistic Toxicity Assessment of Combustible Cigarettes and Oral Products Using HGF Cells

Test Material Name	Figure Representation	Flavor, Nicotine Strength	Pouch Weight	Cytotoxicity[a] (IC_{50})	Oxidative Stress[b]			Inflammation[b]		
					MDA	GSH	GSH/GSSG	IL-8	TIMP-1	MMP-1
3R4F (reference)		NA	NA	Positive (12.01)	↑	↓	↓	↑	↓	↑
1R6F (reference)		NA	NA	Positive (6.76)	↑	↓	↓	↑	↓	↑
CRP1.1 (reference snus)		NA	1.00 g	Negative	↑	NC	NC	↑	↑	↑
CRP2.1 (reference moist snuff)		NA	NA	Positive (67.46)	↑	NC	NC	↑	↑	↑
Commercial NP-1		Smooth, 3 mg	0.40 g	Negative	NC	NC	NC	NC	↑	↑
Commercial NP-2		Coffee, 3mg	0.40 g	Positive (16.62)	NC	NC	NC	NC	NC	↑
Commercial NP-3		Peppermint, 6mg	0.745 g	Negative	↑	NC	↓	↑	NC	NC
Commercial NP-4		Wintergreen, 4mg	0.375 g	Negative	↑	NC	NC	↑	NC	NC
Commercial NP-5		Mint, 2mg	0.220 g	Positive (68.74)	↑	NC	↓	↑	NC	NC
Commercial NP-6		Citrus, 2mg	0.220 g	Negative	↑	NC	↓	↑	NC	NC
Commercial NP-7		Dragon Fruit, 7mg	0.356 g	Negative	↑	NC	↓	NC	NC	↑
Commercial NP-8		Cinnamon, 7mg	0.374 g	Positive	↑	NC	↓	↑	↓	↑

[a] Positive indicates <70% relative viability; IC_{50} not reported if viability is between 50% and 70%.
[b] ↑ (increase) or ↓ (decrease) indicates ≥ 2-fold change relative to concurrent vehicle control; NC indicates no change or <2 fold-change from concurrent vehicle control

Conclusions

This study overall demonstrates that 1)While responses within the NP category varied, all tested oral tobacco products showed substantially lower toxicity, oxidative stress, and inflammation than cigarettes and 2) Primary HGF cells constitute an ideal *in vitro* model for investigation and differentiation of mechanistic toxicity endpoints between tobacco product categories.

References

1. Aldeek, F., McCutcheon, N., Smith, C., Miller, J.H., Danielson, T.L. (2021) Dissolution Testing of Nicotine Release from OTDN Pouches: Product Characterization and Product-to-Product Comparison. Separations 2021, 8, 7.

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