Comparative Toxicity Evaluation of Oral Nicotine Pouch Products to Combustible Cigarettes and Oral Tobacco Comparator Products in Human Gingival Fibroblasts Using In Vitro Mechanistic Assays

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Society of Toxicology 62nd Annual Meeting and ToxExpo
March 19-23, 2023
Abstract ID# 3421; Poster Board number: P545

Abstract

Oral nicotine pouches (NPs) are potential reduced risk alternative tobacco products to cigarettes: They are tobacco leaf-free and thus, most of the harmful and potentially harmful constituents (HPHCs) found in tobacco and tobacco smoke are absent or present at substantially lower levels compared to traditional tobacco products. However, there is limited data on the local toxicity profiles of NPs in comparison with cigarettes and other oral comparators. In this work, we used primary human gingival fibroblasts (HGF) as a clinically relevant in vitro test system to study the potential local toxicity responses following exposure to 12 on!® Test NPs (four on!® NPs [No flavor and three flavors] at three nicotine levels [2, 4 and 8 mg]) and comparator products (combustible reference cigarettes [3R4F and 1R6F], reference snus [CRP1.1], reference moist smokeless tobacco [CRP2.1], four market snus products and six market NPs). Cigarette smoke (CS) condensates were collected in ethanol, using ISO intense puffing regimen, while all oral products (smokeless tobacco products and NPs) were extracted in the enzyme-free artificial saliva (AS) using product to solvent ratio of 10% (w/v). Primary HGF cells were exposed to eight concentrations (up to 333.7 µg/mL nicotine) of each test material for 24 hours. Concentration-dependent decrease in cell viability (IC_{50} <12.0 µg/mL nicotine), induction of oxidative stress (increase in malondialdehyde [MDA] and decrease in glutathione [GSH] and GSH/GSSG) and changes in the levels of inflammatory and tissue damage markers (increase in interleukin-8 [IL-8] and matrix metalloproteinase-1 [MMP-1] and decrease in tissue inhibitor of metalloproteinase-1 [TIMP-1]) were observed in cells exposed to CS. While variable, most oral products exhibited minimal or no cytotoxicity even at higher nicotine concentrations in comparison to CS. All oral products did not exhibit substantial changes in intracellular GSH levels and GSH/GSSG, while some products showed increases in MDA, but to a lesser extent and at higher nicotine concentrations than CS. Most Test and market NPs did not induce notable changes in inflammation and tissue damage markers, while the reference smokeless tobacco products and market snus showed increases in MMP-1 and IL-8, but only at higher nicotine concentrations than CS. In summary, the mechanistic in vitro testing using primary HGF cells demonstrated that the Test NPs have an overall lower or comparable toxic potential compared to other oral tobacco comparator products under the test conditions, while all oral tobacco products exert substantially lower effects on oxidative stress and inflammatory responses compared to cigarettes, supporting their reduced risk potential.

Introduction

Inflammation and oxidative stress are two major mechanisms involved in smoking-related chronic oral diseases, such as periodontitis.[1] NPs, a new form of oral nicotine products, are potential reduced-risk alternatives to conventional cigarettes for adult smokers. These novel products are smokeless and tobacco-free and contain no or lower levels of HPHCs compared to the traditional combustible tobacco products. Since these products are relatively new, their impacts on oral toxicity are not well studied. A comprehensive toxicological assessment of these products using a panel of endpoints relevant to the etiology of oral diseases is necessary to understand their potential toxicity profiles and also in comparison with combustible cigarettes and other oral comparator products. Herein, we provide a summary of a mechanistic screening study where we evaluated the toxicity potential of 12 on!® Test NPs (four on!® NPs, each at three nicotine levels) and compared their toxicity to the combustible cigarettes and oral tobacco comparators (smokeless tobacco including snus and select market NPs) using primary HGF cells and mechanistic in vitro assays (cytotoxicity, oxidative stress and inflammatory response).

Materials and Methods

Mechanistic In vitro Toxicological Assessment Workflow[2]

Test Articles (Table 1)

- **Reference Cigarettes:** 3R4F & 1R6F
- **Smokeless Tobacco (ST) Reference Products:** CRP1.1 & CRP2.1
- **Market Snus:** Four Market snus products
- **Market NPs:** Six Market NPs (comparable flavors to on!® Test NPs)
- **on!® Test NPs:** No flavor and three flavors (Mint, Citrus and Wintergreen) at three nicotine levels of 2, 4 and 8 mg

Test Materials, Test System and Exposure*

- **Cigarette smoke condensates** collected in ethanol, using ISO intense (ISO 20778) puffing regimen
- **Oral product extracts** extracted in enzyme-free Artificial Saliva (AS) at a product to solvent ratio of 10% (w/v).[2] Nicotine concentration in the extract was quantified. Test concentrations were expressed as µg nicotine/mL of AS extract.
- **Primary HGF cells** were exposed to eight concentrations of each Test NP for 24 hours.

*The test materials were prepared by Enthalpy Analytical (Richmond, VA). The in vitro assays were conducted by MB Research Laboratories (Spinnerstown, PA).

In vitro Mechanistic Endpoints

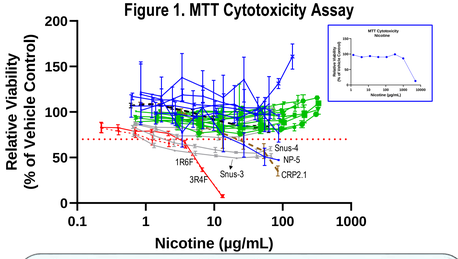
- **Cytotoxicity (MTT)**
- **Oxidative Stress**: MDA, GSH and GSH/GSSG
- **Inflammatory and Tissue Damage Markers:** IL-8, MMP-1 and TIMP-1

Criteria for Evaluating the in vitro Mechanistic Assays

- **Positive**: Cytotoxic, if the relative viability is <70%. **Negative**: Non-cytotoxic, if the relative viability is ≥70% at the highest concentration tested.
- **Outcomes:**
  - **Induction:** minimum effect level (MEL) ≥ 2-fold increase relative to the vehicle controls (indicated by the red dotted lines in the graphs)
  - **Inhibition:** MEL ≥ 50% decrease relative to the vehicle controls (indicated by the red dotted lines in the graphs)
  - **No Change:** If maximum effect level <2-fold increase relative to the vehicle controls; If maximum effect level < 50% decrease relative to the vehicle controls

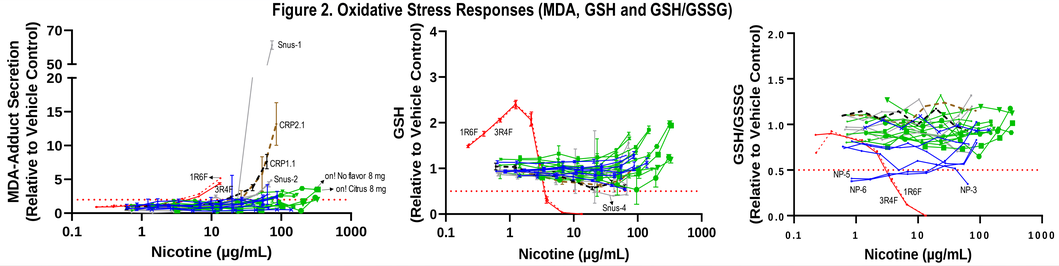
Results

Figure 1. MTT Cytotoxicity Assay



- Combustible reference cigarettes (3R4F and 1R6F) were cytotoxic (a concentration-dependent reduction in viability to <70%; with the IC_{50} < 12.01 µg nicotine/mL).
- ST reference CRP2.1 and some market snus (White and Wintergreen) also decreased viability to <70% and are considered cytotoxic. However, their extrapolated IC_{50}s were 9- to 26-fold higher compared to CS.
- One market NP (Mint 2 mg) was cytotoxic under the test concentrations (IC_{50} = 68.74 µg nicotine/mL). All other oral products - including all on!® Test NPs were not cytotoxic (viability> 70%) even at test concentrations 8- to 36-fold higher than that of CS.
- The observed increase in viability in some oral tobacco products was not likely driven by nicotine (see Inset Figure: MTT cytotoxicity with nicotine only).

Figure 2. Oxidative Stress Responses (MDA, GSH and GSH/GSSG)



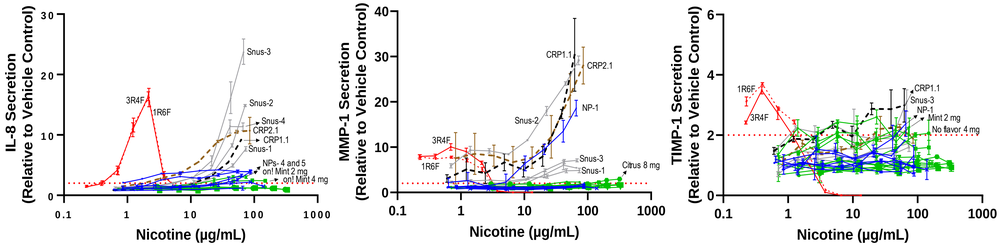
- Combustible reference cigarettes (3R4F and 1R6F) induced oxidative stress in a concentration-dependent manner for MDA-adduct secretion (>4-fold) (Left panel) and a decrease in the intracellular levels of GSH (middle panel) as well as the GSH/GSSG ratio (right panel).
- Among the tested comparator oral tobacco products, all Market snus, CRP1.1 and CRP2.1 induced notable increases in MDA-adduct secretion (between 2- and 62-fold), although mostly at higher nicotine concentrations compared to combustible cigarettes.
- on!® Test NPs (No flavor [2 and 4 mg], Mint [4 mg], Wintergreen [8 mg]) and Market NP (Smooth 3 mg) products did not increase MDA-adduct secretion at all test nicotine concentrations; the rest of the NPs caused ~2-fold induction of MDA-adduct secretion, but only at nicotine concentrations 9- to 64-fold higher than that of CS.
- All on!® Test NPs, most Market NPs (Smooth 3 mg, Cool Mint 6 mg and Wintergreen 4 mg), CRP1.1, CRP2.1 and market snus (except Wintergreen for GSH) had no effects (< 2-fold) on the intracellular GSH level and the GSH/GSSG ratio across the test concentration ranges. Some market NPs (Mint 2 mg, Citrus 2 mg and Peppermint 6 mg) showed limited effects (decreased GSH/GSSG ratio but without affecting the intracellular levels of GSH).

Table 1. Test Products, legends and summary of the in vitro mechanistic study

Test Materials		Nicotine Strength (mg)	Figure Legend	Cytotoxicity[a]	Oxidative Stress[b]			Inflammation[b]		
				IC_{50} (µg nicotine/mL)	MDA	GSH	GSH/GSSG	IL-8	TIMP-1	MMP-1
Reference Cigarettes	3R4F	NA		Positive (12.01)	↑	↓	↓	↑	↓	↑
	1R6F	NA		Positive (6.76)	↑	↓	↓	↑	↓	↑
ST Reference Products	CRP1.1(reference snus)	NA		Negative	↑	NC	NC	↑	↑	↑
	CRP2.1(reference moist snuff)	NA		Positive (67.46)	↑	NC	NC	↑	↑	↑
Market snus-1	No flavor	8.5		Negative	↑	NC	NC	↑	NC	↑
Market snus-2	Mint	8		Negative	↑	NC	NC	↑	NC	↑
Market snus-3	White	8		Positive (37.09)	↑	NC	NC	↑	↑	↑
Market snus-4	Wintergreen	8		Positive (130.86[c])	↑	↓	NC	↑	↑	↑
Market NP-1	Smooth	3		Negative	NC	NC	NC	NC	↑	↑
Market NP-2	Cool Mint	6		Negative	↓	NC	NC	↑	NC	NC
Market NP-3	Peppermint	6		Negative	↑	NC	↓	↑	NC	NC
Market NP-4	Wintergreen	4		Negative	↑	NC	NC	↑	NC	NC
Market NP-5	Mint	2		Positive (68.74)	↑	NC	↓	↑	NC	NC
Market NP-6	Citrus	2		Negative	↑	NC	↓	↑	NC	NC
Test NPs (on!®)	No flavor	2		Negative	NC	NC	NC	NC	↑	NC
		4		Negative	NC	NC	NC	NC	↑	NC
		8		Negative	↑	NC	NC	NC	NC	NC
	Mint	2		Negative	↑	NC	NC	↑	↑	↑
		4		Negative	NC	NC	NC	↑	↑	↑
		8		Negative	↑	NC	NC	NC	NC	NC
	Citrus	2		Negative	↑	NC	NC	NC	NC	NC
		4		Negative	↑	NC	NC	NC	NC	↑
		8		Negative	↑	NC	NC	NC	NC	↑
	Wintergreen	2		Negative	↑	NC	NC	NC	NC	NC
		4		Negative	↑	NC	NC	NC	NC	NC
		8		Negative	NC	NC	NC	NC	NC	NC

[a] Positive refers to cytotoxicity (>30% reduction in viability compared to vehicle control.) [b] ↑ (increase) and ↓ (decrease) indicates ≥ 2-fold induction and ≥ 50% reduction of the endpoint of interest relative to the vehicle control; NC (no change). [c] Extrapolated IC_{50}

Figure 3. Inflammatory Responses (IL-8, MMP-1 and TIMP-1)



- Combustible reference cigarettes (3R4F and 1R6F) induced marked release of IL-8 (>16-fold, left panel) at the non-cytotoxic nicotine concentrations (<2.2 µg/mL). Secretion of IL-8 declined at higher concentrations, at which significant reduction in cell viability was observed. Both reference cigarettes also elicited a concentration-dependent induction of MMP-1 (middle panel), accompanied by inhibition of TIMP-1 secretion (right panel) at the non-cytotoxic nicotine concentrations (0.2 - 2.1 µg/mL), indicating the potential to cause the disruption of extracellular matrix.
- All market snus, CRP1.1 and CRP2.1 increased IL-8 secretion (≥ 7-fold) at nicotine concentrations higher than ~53 µg/mL. All market NPs (except Smooth 3 mg) and on!® NP Mint (2 and 4 mg) induced slight increase in IL-8 secretion, but to a lesser extent (between 2- and 3.5-fold) and at nicotine concentration higher compared to CS.
- All market snus, CRP1.1, CRP2.1 and on!® Test NPs (Mint [2 and 4 mg] and Citrus [4 and 8 mg]) induced MMP-1 secretion but at concentrations >3-fold higher than CS. Except for market NP (Smooth 3 mg; induced MMP-1 and TIMP-1 at >41 µg/mL), all other market NPs did not increase MMP-1 and TIMP-1, even at nicotine concentrations ~700-fold higher than CS.
- Market snus (White and Wintergreen), CRP1.1 and CRP2.1, on!® Test NP No flavor and Mint (2 and 4 mg), and Market NP (Smooth 3 mg) induced the secretion of TIMP-1, while other NPs did not alter TIMP-1 secretion under the test condition.

Conclusion

Our in vitro mechanistic oral health screening assessment of on!® Test NPs and their relative toxicity to cigarettes and oral tobacco comparators shows that:

- on!® Test NPs and all oral tobacco comparator products (CRPs, snus and select market NPs) show notably lower toxic potential for all endpoints (viability, oxidative stress and inflammation) compared to combustible cigarettes under the test condition.
- on!® Test NPs exhibit an overall lower or comparable oxidative stress and inflammatory responses compared to ST products.
- Primary HGF cells serve as one of clinically relevant in vitro test system for investigating and differentiating between tobacco product categories using mechanistic toxicity endpoints.

Taken together, these findings provided additional evidence on the reduced risk potential of the on!® Test NPs relative to combustible cigarettes, supporting their role in tobacco harm reduction.

Limitations

1) Nicotine concentration was used as the dosimetry marker under the assumption that the extraction efficiency for other ingredients (e.g., flavors) is comparable. However, this assumption is not confirmed analytically;
2) These mechanistic in vitro studies were conducted as non-GLP. However, data were checked for accuracy.

References

1. Zhang, Y., He, J., Ge, B., Huang, R., Li, M. (2019) Effect of Tobacco on Periodontal Disease and Oral Cancer. Tobacco induced diseases 17:40.
2. Kumar, A., Doshi, U., Farcas, M., Zhang, M., Marziller, J., DeGeorge, G., Lee, M. (2022). Mechanistic Toxicity Assessment of Oral Nicotine Pouches in Comparison to Combustible Cigarettes and Smokeless Tobacco Using Human Gingival Fibroblasts. 70th TCRC.
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Acknowledgment

We would like to thank Dr. Ashutosh Kumar for technical guidance and study monitoring.

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