Evaluating Toxicity Potential of Oral Nicotine Pouch Products Using in vitro Mechanistic Assays in Primary Human Gingival Fibroblasts

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Abstract

Background and Purpose

Oral nicotine pouches (NPs) are tobacco-free oral nicotine products that present a potentially reduced-risk alternative to tobacco-based products as part of tobacco harm reduction strategies. NPs are free of tobacco leaves and contain nicotine and food-grade ingredients with substantially lower levels of harmful and potentially harmful chemicals compared to combustible cigarette smoke. As a relatively new type of nicotine product, its toxicity profiles are less understood, especially its local (oral) toxicity. In this study, we employed primary human gingival fibroblasts (HGFs) as a clinically relevant in vitro test system. We evaluated a panel of cellular responses relevant to key mechanisms of periodontal diseases[1] using six select market NP products (NP-1 to NP-6) with different nicotine strengths (3-6 mg) and flavor varieties (i.e., cinnamon, fruit, mint, and coffee). Their toxicological effects were compared with those of the combustible 1R6F reference cigarette as well as two reference smokeless tobacco (ST) products (a moist snuff [CRP2.1] and a snus [CRP1.1]).

Methods

Reference cigarettes were smoked using International Organization for Standardization (ISO) intense puffing regimen and smoke condensates were collected in ethanol. All oral products (reference ST products and market NPs) were extracted in enzyme-free artificial saliva (AS) (10% (w/v)). Primary HGFs were seeded on 96-well plates and exposed to eight concentrations (up to nicotine concentrations of 131 µg/mL) of each test material (TM) for 24 hours. Cytotoxicity was assessed using the MTT assay and oxidative stress was evaluated by measuring intracellular levels of glutathione (GSH) and the calculated ratios of GSH/GSSG. Inflammatory response was evaluated using interleukin-8 (IL-8) secretion. IC_{50} values were interpolated using a four-parameter Hill function for cytotoxic TMs. A fold-change threshold of 2.0 was set for GSH and IL-8 and a threshold of 0.5 for GSH/GSSG ratios for positive responses. Threshold concentrations for each endpoint were calculated using a four-parameter logistic (4PL) regression and compared to that of 1R6F (smoke condensate).

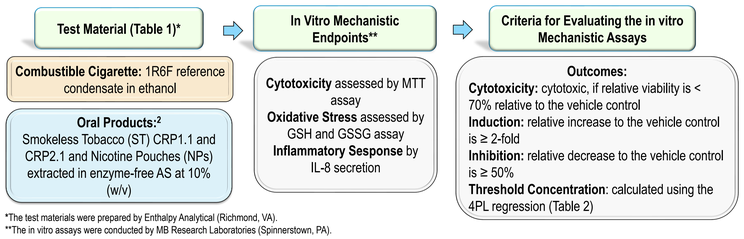
Results

1R6F smoke condensates showed a clear concentration-dependent decrease in cell viability (IC_{50} < 8.0 µg/mL nicotine), induction of oxidative stress (an increase in GSH levels and a concurrent decrease in the GSH/GSSG ratio), and inflammatory response (an increase in IL-8). Tested oral NP products, except for NP-1 (IC_{50} of 27.07 µg/mL), were noncytotoxic even when tested at higher nicotine equivalent concentrations (approximately 30-fold higher) than 1R6F. NP-1 & 3 induced an increase in GSH levels and concurrently a decrease in the ratio of GSH/GSSG, suggesting disruption of GSH homeostasis and thus induction of oxidative stress. ST products (CRP1.1 & CRP2.1), NP-1 & 3 induced notable increases (>3-fold increase) in IL-8 secretion. These results suggest differences in toxicity potential among oral products and flavor variants, however all NPs were substantially and consistently less toxic (i.e., the threshold concentrations were approximately 2- to 13-fold higher) compared to 1R6F under the testing conditions.

Conclusions

In summary, our mechanistic in vitro testing using primary HGF cells was able to differentiate in vitro toxicity responses related to key mechanisms of oral diseases among different categories of tobacco products. Although flavor-related toxicity was observed under the testing condition, the in vitro toxicity of these test NPs was consistently lower compared to combustible cigarettes, supporting the reduced risk potential of the NPs and their role in tobacco harm reduction.

Materials and Methods



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

Results

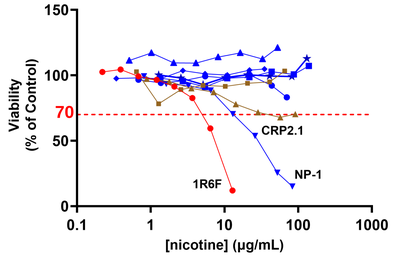


Figure 1. Cytotoxicity by MTT.
Reference 1R6F cigarette and NP-1 showed concentration-dependent decrease in viability. CRP2.1 exhibited moderate cytotoxic, with the lowest cell viability of approximately 70%.

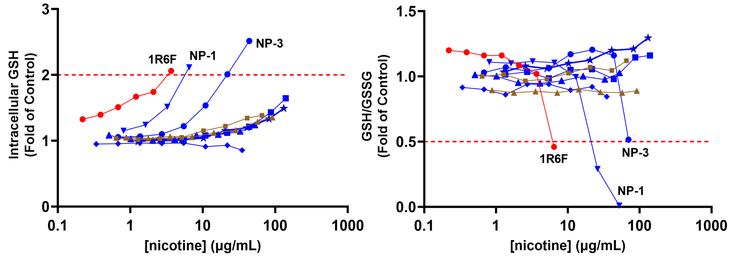


Figure 2. Assessment of oxidative stress by GSH and GSSG assay.
1R6F cigarette, NP-1, and NP-3 induced oxidative stress as demonstrated by increase in GSH (left panel) and decrease in GSH/GSSG ratio (right panel).

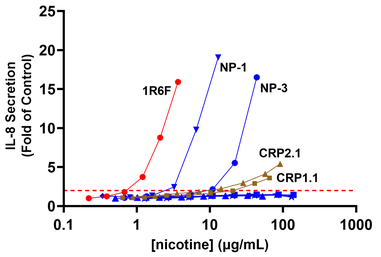


Figure 3. Induction of inflammatory response (IL-8).
Reference 1R6F cigarette, CRP1.1, CRP2.1, NP-1, and NP-3 induced marked IL-8 release at non-cytotoxic concentrations.

Table 1. Summary of Tested Products

Test Products (and flavor variant for each NP)		Figure Legend	Test Concentration (µg nicotine/mL)
Reference Cigarette	1R6F		0.22-12.85
ST Reference Products	CRP1.1 (reference snus)		0.64-65.59
	CRP2.1 (reference moist snuff)		0.89-91.23
Market NP-1	Cinnamon, 6 mg		0.81-83.17
Market NP-2	Mango, 3 mg		0.32-32.42
Market NP-3	Cinnamon, 3 mg		0.68-70.11
Market NP-4	Coffee, 6 mg		1.36-139.37
Market NP-5	Mint, 6 mg		1.28-130.96
Market NP-6	Coffee, 4 mg		0.51-52.41

Table 2. Summary of the Responses of the Tested Products

TMs	IC_{50} (µg nicotine/mL)	Threshold concentration (µg/mL nicotine)			Fold Change Relative to 1R6F*			
		GSH	GSH/GSSG	IL-8	MTT	GSH	GSH/GSSG	IL-8
1R6F	7.7	3.37	6.29	0.76	1.0	1.0	1.0	1.0
CRP1.1	Not cytotoxic	No change	No change	18.58	-	-	-	24.5
CRP2.1	Not cytotoxic	No change	No change	10.24	-	-	-	13.5
NP-1	27.06	5.81	21.1	2.83	3.5	1.7	3.4	3.7
NP-2	Not cytotoxic	No change	No change	No change	-	-	-	-
NP-3	Not cytotoxic	21.43	70.17	9.82	-	6.4	11.2	12.9
NP-4	Not cytotoxic	No change	No change	No change	-	-	-	-
NP-5	Not cytotoxic	No change	No change	No change	-	-	-	-
NP-6	Not cytotoxic	No change	No change	No change	-	-	-	-

* Fold change = threshold concentration$_{NP}$/threshold concentration$_{1R6F}$

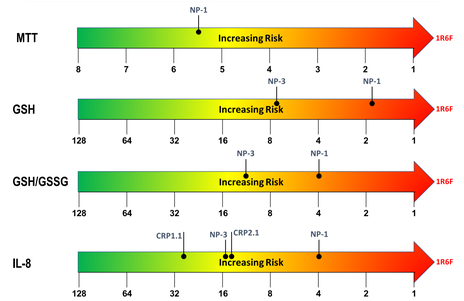


Figure 4. Fold changes of CRPs and NPs relative to 1R6F. Threshold concentrations for each endpoint were calculated (Table 2) and fold change relative to 1R6F was displayed for in vitro-responding products only (CRP1.1 & 2.1; NP-1 & 3)

Conclusion

Our in vitro mechanistic toxicity screening was able to demonstrate differential (reduced) toxicity potential of ST and NPs relative to combustible cigarettes, based on the in vitro responses related to the known modes-of-action for oral health toxicity (viability, oxidative stress, and inflammation).

- Combustible cigarettes showed clear increase in toxicity with all endpoints and at relatively lower exposure concentrations compared to ST and NPs.
- Among tested NPs, two NPs showed notable responses, while the rest of the four NPs showed no toxicity responses. NP-1 & 3 (cinnamon-flavored, 6 & 3 mg nicotine, respectively) triggered both oxidative stress and inflammatory responses at non-cytotoxic concentrations. However, these responses were observed at consistently higher exposure levels compared to the combustible cigarettes.
- For ST, CRP1.1 and CRP2.1 elicited lower levels of inflammatory responses at high exposure concentrations compared to 1R6F, NP-1, and NP-3, without altering GSH homeostasis.

Taken together, the findings reported here support the harm reduction potential of the oral nicotine products relative to combustible cigarettes.

Limitations

1) Limited oxidative stress and inflammatory response endpoints were measured in this study. Integration of additional assays is may be necessary to fully understand the effects of these novel oral nicotine products on these key responses.
2) This was a preliminary assessment, only limited market NPs (in terms of range of flavors, nicotine strengths, and manufacturers) were tested. The results may not be representative of the entire NP category.

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. 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 8:7.

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