Pre-Clinical in vitro assessment of tobacco-free nicotine pouch products

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INTRODUCTION

Smoking combustible tobacco products like cigarettes is known to cause serious disease in smokers, including lung cancer, heart disease and emphysema. Recent advances in technology have, and continue to, facilitate the creation of a range of new and innovative alternative nicotine products, which either do not combust tobacco or do not contain tobacco leaf, and have the potential to be substantially less harmful for adult smokers than continued combustible cigarette smoking. Tobacco-free nicotine pouches (TFNPs) are a recent product innovation available in a growing number of countries, adding to the repertoire of potentially reduced harm alternatives for adult smokers who would otherwise continue to smoke. TFNP has a similar appearance and oral route of use to that of traditional tobacco-containing Scandinavian Snus. Typically, TFNPs are small pouches containing high-purity pharmaceutical-grade nicotine within a plant fibre-based substrate alongside other high-quality ingredients such as flavourings.

AIM

In the current study we characterised the *in vitro* toxicological activity of extracts from two commercially available TFNPs, one snus product and where relevant compared the biological response to total particulate matter (TPM) from 1R6F Reference Cigarette on a nicotine basis.

METHODS

Test Articles

Product type	Coding	Primary substrate	Nicotine content
TFNP	TFNP #2	Plant fibres	5.8 mg/pouch
TFNP	TFNP #3	Plant fibres	10.1 mg/pouch
Skandinavian Snus	Snus	Tobacco leaf	10.9 mg/pouch
Combustible cigarette	1R6F	Tobacco leaf	0.7 mg/cigarette

Generation of TFNP/ Snus extracts

Extraction of TFNP/snus was based on ISO 10993-12. In brief, 6g of product was covered with 20ml PBS as extraction medium to obtain an extraction ratio of 300mg/ml and agitated at 600rpm and 37°C for 1 hour. After centrifugation and filtration through 0.45 and 0.2µm sterile filters, aliquots were stored frozen at -80°C. Nicotine content was determined using LC-MS/MS.

In vitro assays

The Neutral red uptake (NRU) was performed to determine cytotoxicity in BEAS-2B (ECACC;Cat.No.:95102433) and HepG2 (ATCC,Cat.No.:HB8065) cells, following standard assay protocols in accordance with ISO 17025; *Salmonella typhimurium* reverse mutation assay (Ames test) for mutagenicity in TA98, TA100, TA102, TA1535 and TA1537 in compliance with OECD test Guideline 471; and *in vitro* micronucleus (IVM) with V79 (ECACC;Cat.No.:86041102) for genotoxicity in compliance with OECD test Guideline 487.
Skin sensitising hazard properties were determined using the GARDskin assay with the endpoints cytotoxicity (PI staining) and RNA analysis of skin sensitiser specific biomarkers.
3D human oral epithelium tissue for was used to assess the mucosal irritation potential via cytotoxicity (MTT), barrier integrity (TEER), cytokine quantification (IL-1α , IL-6 and CCL-20) and histology (H&E staining and TUNEL assay).
Cytotoxicity on EpiGingival Tissue Models (MatTek) was measured via MTT staining and measurement of LDH release.

RESULTS

Nicotine quantification of PBS extracts/ TPM

The concentration of nicotine in the test articles extracts is shown on the right. Snus extracts contained the highest level of nicotine per millilitres, whereas 1R6F TPM contained the lowest.

Product extracted	mg nicotine/ ml solvent
TFNP #2	1.70 mg/ml PBS
TFNP #3	2.51 mg/ml PBS
Snus	2.59 mg/ml PBS
1R6F TPM	1.35 mg/ml DMSO

Neutral Red Uptake Cytotoxicity assay

The 1R6F TPM, TFNPs and snus extracts all showed statistically significant cytotoxicity in both cell lines HepG2 and BEAS-2B, indicated by cytotoxicity values >20% (EC20) within the tested concentration range. 50% cytotoxicity (EC_{50}) was not reached by TFNPs, whereas the snus extract achieved EC_{50} in HepG2 cells.
The table below displays the EC_{20} and EC_{50} values with corresponding confidence intervals (CI) and compared to 1R6F TPM on nicotine basis. All OND products demonstrated marked cytotoxicity reductions compared to TPM on a per nicotine basis as indicated by significantly increased EC_{20}/EC_{50} values[1].

Cell line	Sample ID	EC_{50} (µg/ml nicotine)	95% CI From	95% CI To	Fold reduction compared to 1R6F	EC_{20} (µg/ml nicotine)	95% CI From	95% CI To	Fold reduction compared to 1R6F
BEAS-2B	TFNP #2	475*	305	868.1	549	35.8	31.5	41.6	79
HepG2		113*	92	147	83	17.1	14.8	19.4	24
BEAS-2B	TFNP #3	890*	577	1576	1029	39.9	35.4	45.4	88
HepG2		111*	98	131	82	26.9	23.8	30.1	37
BEAS-2B	Snus	192*	168.5	225.2	222	55.2	52.2	58.3	122
HepG2		38.6	37.4	39.8	28	20.2	19.1	21.2	28
BEAS-2B	1R6F TPM	0.865	0.831	0.900	1	0.453	0.413	0.494	1
HepG2		1.36	1.32	1.39	1	0.722	0.685	0.758	1

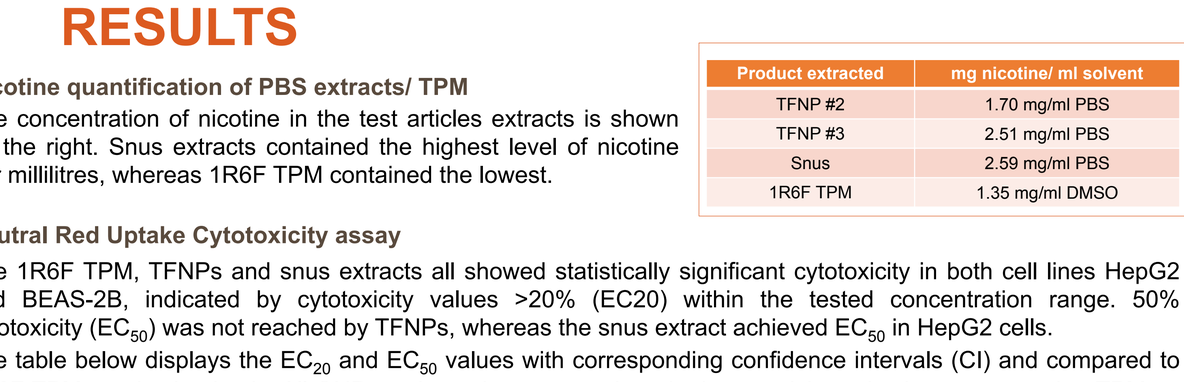

Figure 1: Percent cytotoxicity induced by increasing concentrations of 1R6F TPM, TFNPs and Snus extracts and neat nicotine in the NRU assay with BEAS-2B and HepG2 cells. The grey dotted line indicates 50% cytotoxicity (EC_{50}). Results of three independent replicates ± SEM.

Local tolerance on 3D oral mucosa (VitroScreen S.r.l.)

EPISKIN® Reconstituted human oral epithelium were treated with TFNPs/ snus extracts (1560mg/l) and neat nicotine (1560mg/ml) for 1h. No signs of cytotoxicity (MTT, LDH, TUNEL) and no impact on the epithelial permeability and physiological homeostasis (TEER) was observed. The expression of the pro-inflammatory cytokines IL-1α, IL-6 and CCL-20 was not changed by the TFNPs, snus and neat nicotine.

RESULTS CONTINUED

Salmonella typhimurium reverse mutation assay (Ames test)

TPM from 1R6F reference cigarette caused a dose dependent and statistically significant increase of revertants in TA98±S9, TA100±S9 (See Figure 2) and TA1537+S9 (data not shown). By contrast, no dose-dependent or statistically significant increases in revertant frequencies were observed for TFNPs and snus extracts in any of the five *S. typhimurium* strains (TA98, TA100, TA102, TA1535, and TA1537) with or without S9 metabolic activation, when compared with the negative controls[1].

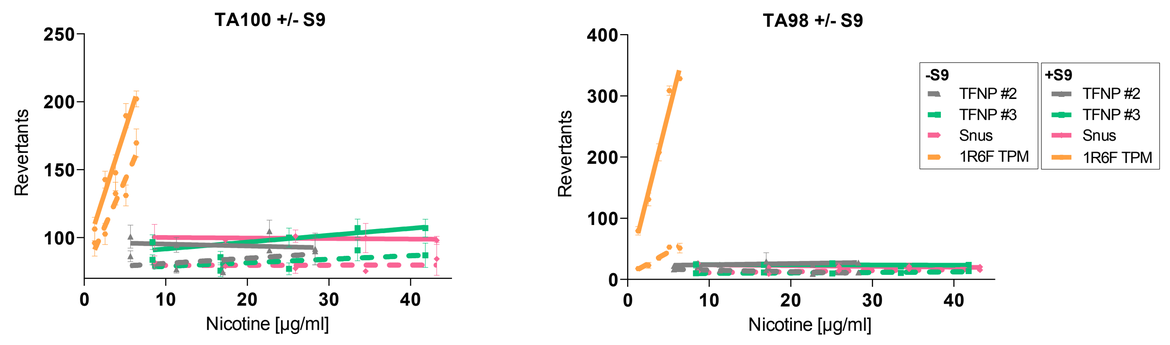

Figure 2: Does-specific mutagenicity in *S. typhimurium* TA98 and TA100 with and without metabolic activation (S9) after exposure to TFNPs, snus and reference cigarette TPM. Results of two independent replicates ± SEM.

In vitro micronucleus genotoxicity assay

TPM from 1R6F reference cigarette caused dose dependent and statistically significant increases in micronucleus frequencies in all three treatments: short-term (3.5h; ST) ±S9 and long-term (22±2h; LT) -S9, indicating a strong genotoxic response. At the top testing concentration the relative population doubling (RPD) decreased, indicating cytotoxicity levels between 45% (ST+S9), 47% (LT-S9) and 88% (ST-S9). No signs of cytotoxicity and no-dose dependent increases in micronucleus frequencies were observed for the TFNPs or snus extracts[1].

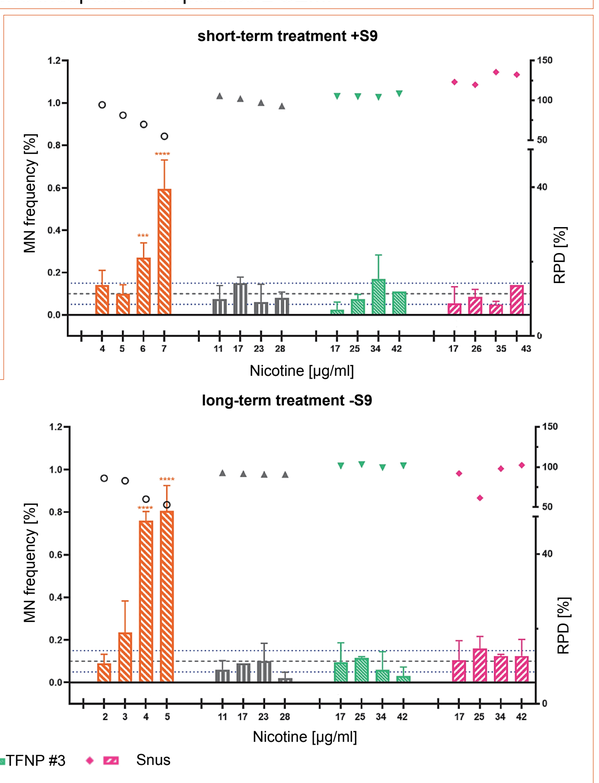

Figure 3: Relative micronucleus frequencies (%), with overlay of RPD values. Background micronucleus frequencies indicated by the dashed line, with dotted line showing upper and lower SD. Orange shaded area in ST-S9 (lower left) indicates RPD less than 40%.
**p < 0.01, ***p < 0.005, ****p < 0.0001. Results of two independent replicates ± SD.

EpiGingival Tissue Cytotoxicity

EpiGingival Tissue Models (MatTek) were exposed to undiluted TFNPs and snus extracts for 4h (ST) and 16h (LT). Statistically significant increase of LDH release compared to the negative control (solvent) was observed after long treatment with TFNP#3 and snus as a result of cell lysis. The cytotoxic effect of snus was reproducible via MTT staining, revealing a significant decrease of the cell metabolism after long treatment with the product.

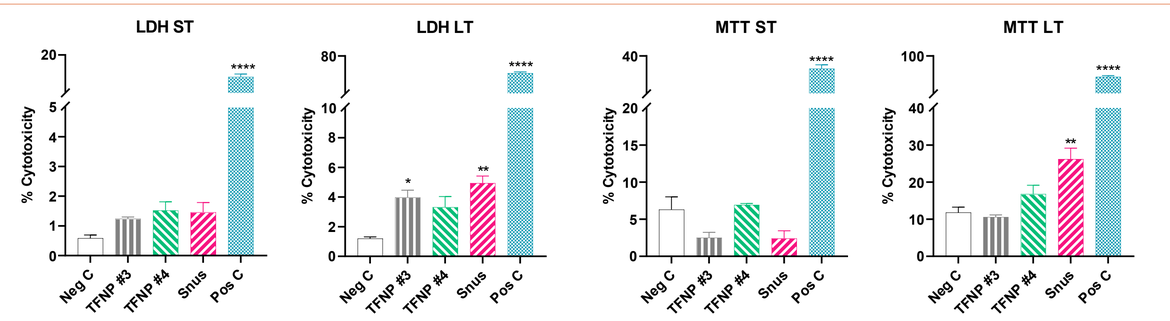

Figure 4: % cytotoxicity calculation of the optical densities from the LDH and MTT assay after short (ST) and long (LT) treatment with PBS extracts from TFNPs and snus.
* p<0.05, ** p<0.01, **** p<0.0001 vs. negative control (Neg C); 1% Triton-X-100 served as positive control (Pos C).

Genomic Allergen Rapid Detection™ (GARDskin) assay for skin sensitization (©SenzaGen)

Human dendritic-like cells (SenzaCells™) were treated with TFNPs/ snus extracts with 500µM nicotine for 24h. No cytotoxicity was observed. Genomic readout of 200 genes relevant to sensitization led to the classification of the snus product as “skin sensitizer” whereas the TFNPs were classified as “skin non-sensitizer”.

4. CONCLUSIONS

The tested TFNPs and snus have a substantially reduced *in vitro* toxicity activity compared with 1R6F reference cigarette. These findings add to the available body of scientific evidence that TFNPs and snus are a less harmful alternative to combustible cigarettes. Especially the assessed TFNPs offer promising tobacco harm reduction potential when used by adult smokers as an alternative to smoking combustible cigarettes.
These findings will need to be further substantiated in studies including advanced *in vitro* techniques and clinical assessments.

REFERENCES

1. Yu *et al*., Preclinical Assessment of Tobacco-Free Nicotine Pouches Demonstrates Reduced In Vitro Toxicity Compared with Tobacco Snus and Combustible Cigarette Smoke. Applied In Vitro Toxicology.Mar 2022.24-35.
2. Stevenson *et al*.. The use of Genomic Allergen Rapid Detection (GARD) assays to predict the respiratory and skin sensitising potential of e-liquids. Regul Toxicol Pharmacol. 2019 Apr;103:158-165.