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In vitro assessment of Tobacco-Free Nicotine Pouches reveals marked reductions in toxicity when compared to cigarettes

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INTRODUCTION

Tobacco-Free Nicotine Pouches (TFNPs) are a category of nicotine-containing oral products that deliver nicotine orally via the gum mucosa. These products do not contain tobacco leaves and do not undergo combustion. A growing body of evidence shows that TFNP extracts contain substantially fewer and lower levels of toxicants compared to cigarettes.[1,3,4,5] Studies have shown that lower levels of toxicants translate to reduced in vitro biological activity in TFNP extracts compared to cigarette smoke extracts. In this study, we tested a variety of Zone™ TFNPs, commercially available in the UK, using three in vitro toxicological assays to demonstrate their reduced risk potential when compared to a cigarette smoke extract. The tested products cover four flavour directions and nicotine strengths: Menthol (11 mg nicotine/pouch), Citrus (12 mg nicotine/pouch), Berry (14 mg nicotine/pouch), and Mint (20mg nicotine/pouch).

METHODS

Generation of PBS Extracts

The extraction of TFNP pouches was performed in accordance with ISO 10993-12:2021, chapter 10. Each 6g of TFNP was covered with 20 ml of phosphate-buffered saline (PBS) to obtain an extraction ratio of 300mg/ml, then agitated at 600rpm at room temperature for 1 hour. After centrifugation and filtration through 0.2 µm sterile filters, 550 µl aliquots per extract were frozen at -80°C.

Biological Assessment

The in vitro techniques were performed according to the methodology outlined by Yu et al.[2] Statistical analyses were performed using GraphPad Prism version 10.6.1

NRU

The NRU assay was carried out in BEAS-2B and HepG2 cells, alongside negative and positive controls; TFNP extracts were added to cultures at concentrations ranging from 0.5 to 21mg/ml medium. 1R6F TPM (Total Particulate Matter) was applied in the range of 0.005-0.05mg/ml medium. Outcomes were compared based on the concentrations required to induce 20% (EC_{20}) and 50% (EC_{50}) cytotoxicity. A comparison was made between 1R6F and TFNP using ANOVA and Dunnett's tests. Pairwise comparisons using Tukey's test were carried out among nicotine pouch products.

In Vitro Micronucleus (IVM)

Performed according to OECD Guideline 487 - Three treatment schedules were applied in the micronucleus assay to Chinese hamster lung fibroblast V79 cells: short-term (ST) +S9, short-term (ST) –S9 and long-term (LT) –S9. The treatment schedules for 1R6F TPM and Menthol TFNP extract were applied to human lymphoblastoid TK6 cells. For the TFNP extracts, cells were exposed to a range of concentrations between 2-5mg/ml medium, and for 1R6F TPM, this range was 0.008-0.085mg/ml medium (tested alongside negative and positive controls). Outcomes were assessed for significance using chi-square pairwise comparisons and a trend test in V79 cells and one-way ANOVA with comparisons against the negative control and a one-way ANOVA trend test in TK6 cells.

Ames

Performed according to OECD Guideline 471 - Five Salmonella typhimurium strains were assessed in the bacterial reverse mutation (Ames) test, TA98, TA100, TA102, TA1535 and TA1537 (+/-S9). Alongside negative and positive controls, TFNP extracts were added to cultures at concentrations ranging from 1-5mg/plate; 1R6F TPM was obtained under ISO conditions and applied at concentrations ranging from 0.025-0.125mg/plate. Mutagenic activity was analysed by fitting a non-threshold model to the dose-response slope (fold increase in revertants) and applying Dunnett's test.

RESULTS

NRU

EC_{20} values obtained for TFNP extracts were 16 to 71 times less cytotoxic than the 1R6F cigarette TPM when calculated per nicotine basis (Figure 1).

EC_{50} values obtained for TFNP extracts were 53 to 215 times less cytotoxic than the 1R6F cigarette TPM when calculated per nicotine basis.

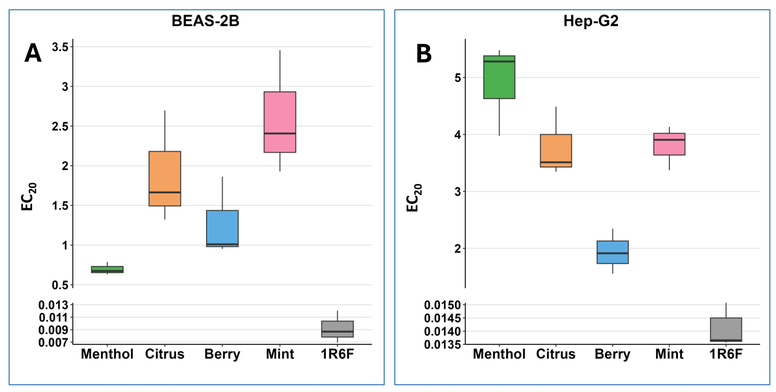

Figure 1 (A-B): Concentrations (mg extract/ml medium) required to induce 20% (EC_{20}) cytotoxicity in HepG2 and Beas-2B cells compared to the negative control.

IVM

None of the TFNP extracts induced dose-dependent, reproducible, or statistically significant increases in micronucleus frequencies compared to the negative controls in any of the three treatment schedules applied; 1R6F TPM induced significant, dose-dependent, and reproducible increases in micronucleus frequencies as observed in Figure 2.

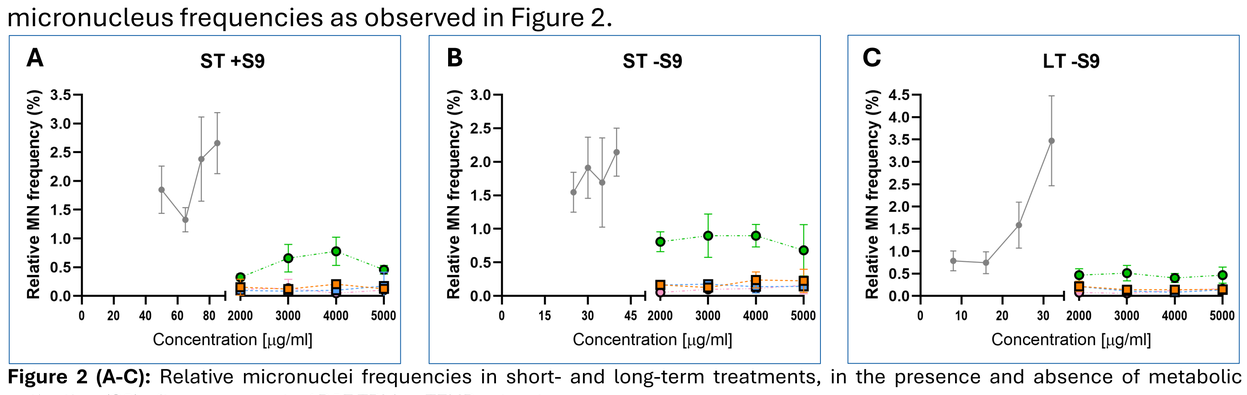

Figure 2 (A-C): Relative micronuclei frequencies in short- and long-term treatments, in the presence and absence of metabolic activation (S9) after exposure to 1R6F TPM or TFNP extracts.

Ames

None of the TFNP extracts demonstrated any evidence of causing reproducible, dose-dependent, or statistically significant increases in the number of revertants; 1R6F TPM caused reproducible, dose-dependent, statistically significant increases in the number of revertants in TA98 (+/-S9), TA100 (+/-S9) and TA1537 (+S9) as observed in Figure 3.

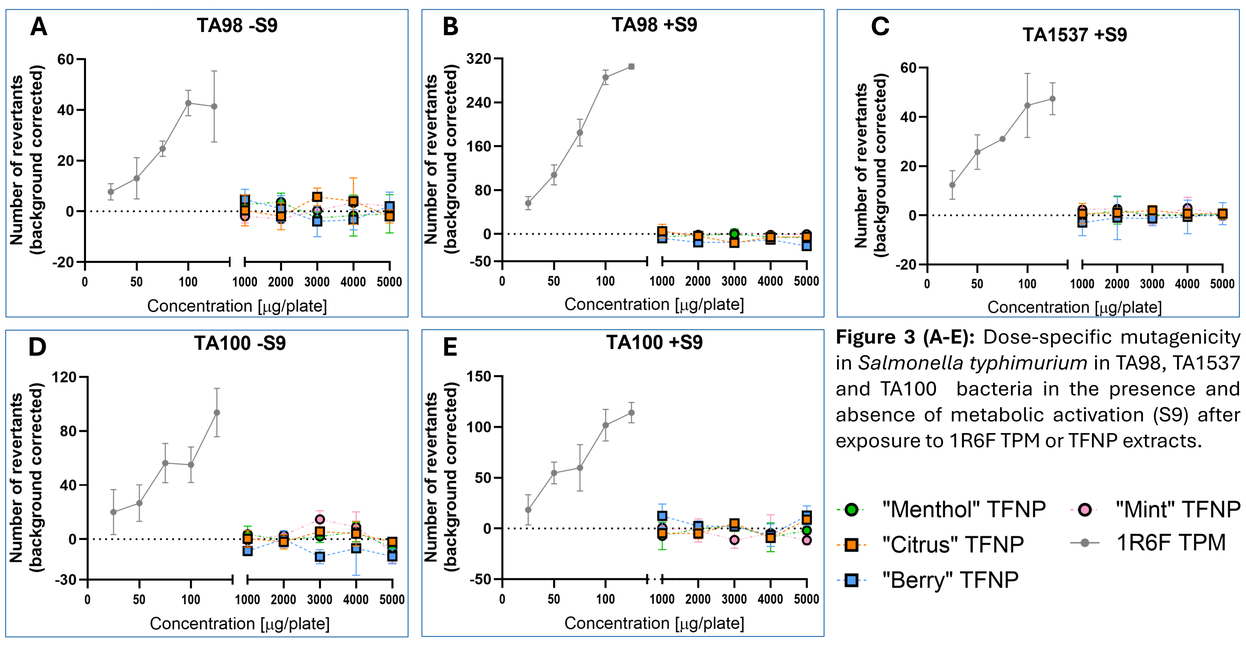

Figure 3 (A-E): Dose-specific mutagenicity in *Salmonella typhimurium* in TA98, TA1537 and TA100 bacteria in the presence and absence of metabolic activation (S9) after exposure to 1R6F TPM or TFNP extracts.

CONCLUSIONS

In the NRU assay, 1R6F TPM induced higher cytotoxicity compared to TFNP extracts in HepG2 and BEAS-2B cell lines by 16 to 215-fold. No correlation was observed between cytotoxicity and TFNP nicotine content in the NRU assay for either cell line.

In the IVM and Ames assays, the TFNP extracts showed no evidence of genotoxicity or mutagenicity under the test conditions, whereas 1R6F TPM met the criteria to be classified as genotoxic and mutagenic.

These results highlight that the lower toxicant levels in these particular TFNPs, compared to cigarette smoke, lead to marked reductions in in vitro activity. This suggests that TFNPs have the potential to offer a harm-reduced alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction.

Public health bodies have concluded that nicotine is addictive. However, they also agree that the smoke created from burning tobacco leaf contains the harmful chemicals that are responsible for smoking-related diseases[6]. The TFNPs in this study contain nicotine but not tobacco leaf, enabling adults to consume nicotine without tobacco or the generation of smoke through tobacco combustion.

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