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Assessment of the potential biological effects of Tobacco-Free Nicotine Pouches using New Approach Methodologies

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INTRODUCTION

Tobacco-Free Nicotine Pouches (TFNPs) do not burn tobacco and do not contain any tobacco leaf. Research demonstrates they contain significantly fewer and substantially lower levels of harmful chemicals compared to snus or cigarette smoke. To determine if this reduction of toxicants translates into reduced harm, a selection of New Approach Methodologies (NAMs) were employed, assessing a range of cellular endpoints. TFNP and tobacco-based Snus were obtained from the European market and compared to reference cigarette smoke extracts. Snus and TFNPs were extracted using ISO-10993-12 methodology, whilst cigarette smoke extracts were generated via bubbling through cell media/PBS or via DMSO extraction of Cambridge filter pads (e.g., Total Particulate Matter-TPM). Levels of nicotine in the extracts were used as a marker of dosimetry, allowing for comparisons of response across the product categories. Our assessments utilised high-content screening technology of endothelial cells and an oral-immune cell coculture system. The endpoints assessed were cellular stress and death, DNA damage, inflammatory marker release, oxidative stress and macrophage morphology.

METHODS

2.1 Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- Snus, Skruf No2 fresh S2 (10mg/pouch nicotine)
- TFNP, Skruf Super White No52 Fresh S2 (5.8mg/pouch nicotine)

Both smokeless products were obtained from the EU market.

2.2 OND Extract Preparation

Snus and TFNPs pouches were extracted following the method outlined by Yu *et al.* (2022), utilising EGM-MV2 medium for the Human Coronary Artery Endothelial Cells (HCAEC) test and PBS for the oral-immune coculture test. Nicotine content was quantified, and extracts were stored at −70 ± 6 °C. Cell media extracts allowed for higher test exposures (i.e. TFNP up to 95%) compared to PBS extracts (up to 10%).

2.3 Total particulate matter (TPM) and Smoke bubbled media (SbMed)/PBS Extracts

1R6F reference cigarettes were smoked under Health Canada Intense conditions. TPM was collected on Cambridge filter pads and extracted with DMSO. For smoke bubbled (Sb) extracts, smoke was bubbled through either PBS or cell culture medium (adapted from Czekala *et al.*, 2020). Nicotine content was quantified, and extracts were stored at −70 ± 6 °C.

2.4 Cell Culture

Primary HCAECs (Promocell C-12221; male, 55, never-smoker) were cultured in coated T175 flasks and seeded into collagen-coated 96-well plates (100 µL of $1.2{\times}10^5$ cells/mL). After overnight adherence, cells were exposed for 4 or 24 h with or without NAC. Extract concentrations and osmolarities are detailed in Table 1. With regards to the oral-immune coculture, ImmuPHAGE™ was seeded onto black µ-clear 24-well plates at a density of 2.5×10^5 viable cells/mL (500 µL/well) and incubated at 37°C in an atmosphere of 5% CO_2 (standard conditions) until required. On the day of dosing, the TR146 cells on Transwell® inserts (supplied by SkinEthic™) were combined with wells containing ImmuPHAGE™ cells. The biological assessment of oral-immune coculture was performed by ImmuONE Ltd.

2.5 Immunostaining of HCAEC

Post-exposure, cells were fixed (4% formaldehyde), permeabilized (0.1% Triton X-100), and blocked in PBS/BSA. Primary antibodies (1:250–1:500) targeted p-c-JUN, SRXN1, NFκB, and γH2AX. Nuclei were counterstained with DAPI. Imaging and quantification were performed using the CELLINSIGHT CX5 High Content Screener (Thermo). Cell counts and nuclear size (via DAPI) were analysed. Fluorescence intensities for p-c-JUN, SRXN1, NFκB, and γH2AX were used to calculate nicotine-based minimum effective concentrations (MECs), visualized as pie charts using Gladiatox (Belcastro et al., 2019).

2.6 Caspase 3/7 Assays of HCAEC

Caspase 3/7 activity was assessed using CellEvent Green Detection Reagent (C-10423). Hoechst 33342 was used for nuclear counterstaining. Fluorescence was measured post-fixation on the High Content Screener. Apoptosis dose-response data was analysed in GraphPad Prism (v10.3.1).

2.7 Immunostaining of Immune cells

ImmuPHAGE™ cells were stained with a dye cocktail containing Hoechst 33342 (nuclei), MitoTracker Red (active mitochondria), caspase 3/7 (early apoptosis) and imaged immediately, followed by fixing and staining with Cell Mask Deep Red (cytoplasm to identify vacuoles). Images were acquired using an InCell Analyser 6000 system (GE Healthcare). Each sample was imaged using 36 fields representing in total between 500 to 1500 cells per well. Images were analysed and quantified for cell morphology (cell area, number of vacuoles and total area of vacuolation) and cell health (mitochondrial and caspase 3/7 activity).

2.8 PrestoBlue™ Cell Viability of TR146

Cell viability was measured using PrestoBlue reagent, which detects metabolic activity via resazurin reduction. Transwell® inserts were incubated with 180 µL medium and 20 µL PrestoBlue at room temperature for 2 h. Fluorescence was read at 520 nm excitation and 580–640 nm emission using a Promega GloMax™ plate reader.

RESULTS

3.1 Human Coronary Endothelial Cells High Content Screening

Apoptosis

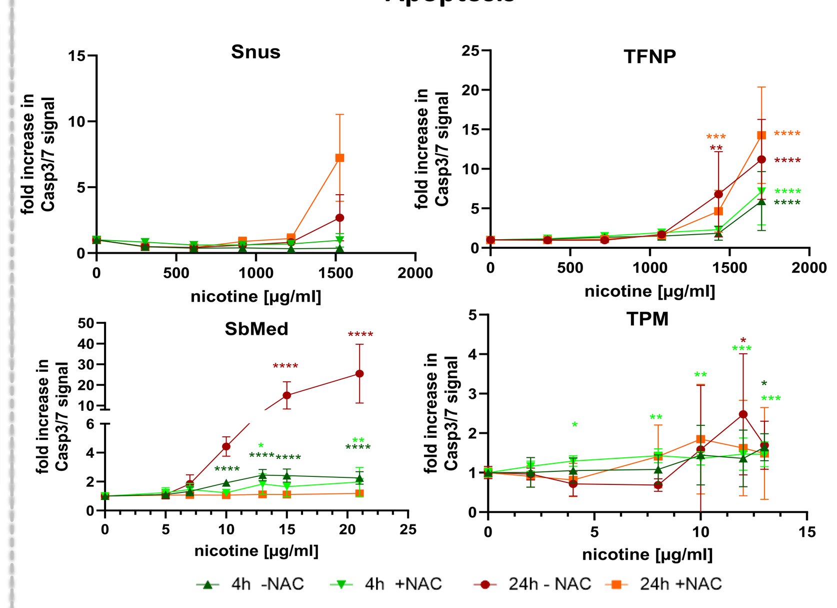


Figure 1: Induction of apoptosis following 4h and 24 hours of exposure of HCAECs to the different extracts +/- NAC. For Snus and TFNP extracts an induction of apoptosis was observed at dose levels that also exhibited increased osmolarity values and beyond those of MEC for nuclear size indicating the induction of apoptosis (Table 1). For 1R6F SbMed, apoptosis was largely driven by oxidative stress which was especially obvious after 24hours of exposure, while 16RF TPM induced a limited though statistically increase without clear difference regarding NAC presence. *Significance: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

SNUS medium extract			TFNP medium extract			1R6F TPM in DMSO			1R6F SbMED		
% extract	nic [µg/ml]	Osmolarity	% extract	nic [µg/ml]	Osmolarity	µg/ml	nic [µg/ml]	Osmolarity	% SbMED	nic [µg/ml]	Osmolarity
0	0	0,301	0	0	0,296	0	0	0,413	0	0	0,385
8	305	0,374	20	330	0,438	40	2	0,416	2	5	0,373
16	610	0,455	40	659	0,581	80	4	0,416	3	7	0,364
24	916	0,536	60	989	0,716	150	8	0,41	4	10	0,367
32	1221	0,604	80	1318	0,865	200	10	0,412	5	13	0,365
40	1526	0,677	95	1565	0,973	240	12	0,415	6	15	0,362
						260	13	0,405	8	21	0,366

Table 1: Applied dosing for the different extracts in % extract and the calculated nicotine concentrations therein. As a separate measure the osmolarity values (osml/L) are added. The results indicate a high osmolar activity of the Snus/ TFNP samples.

Cellular stress summary

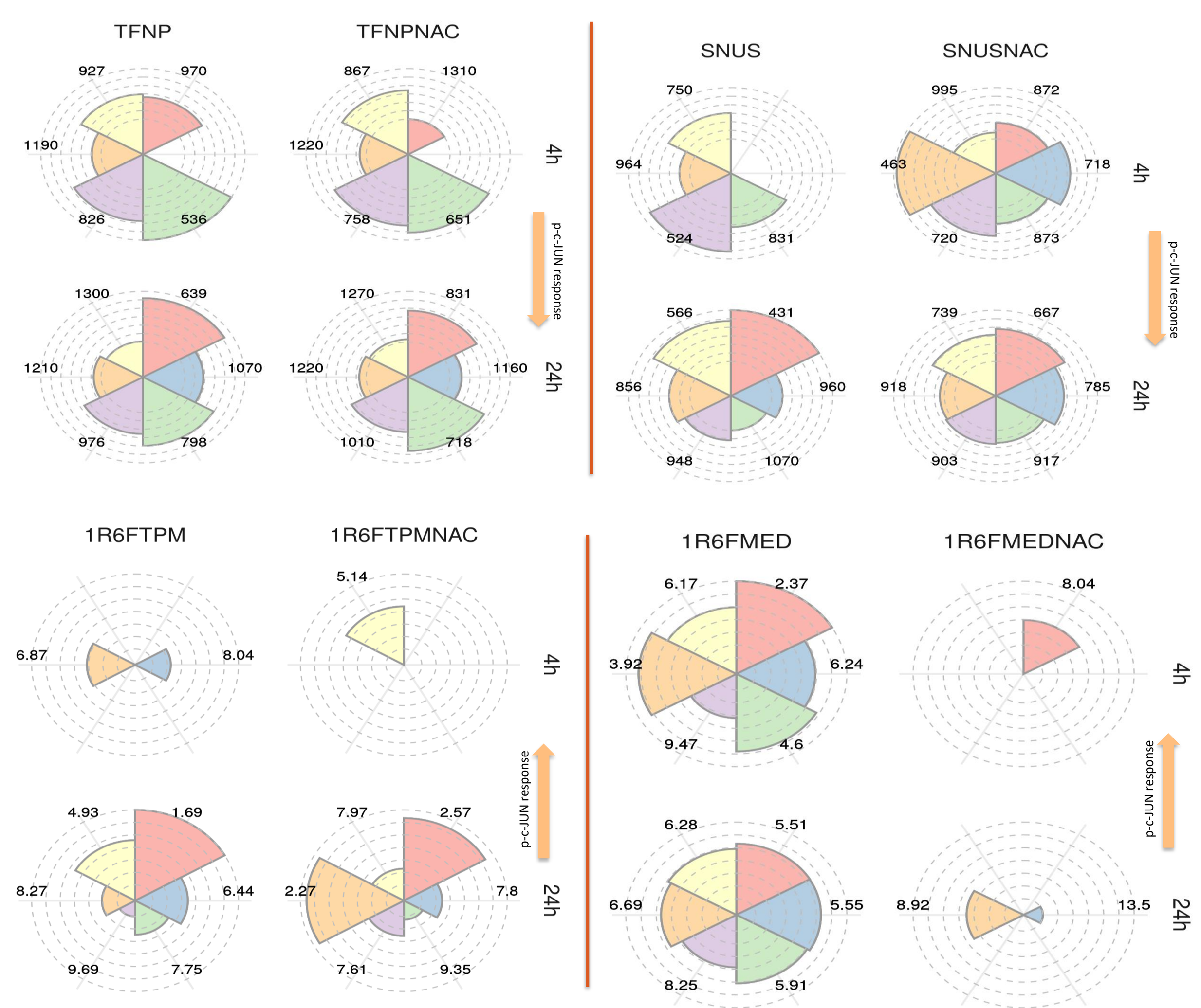


Figure 2: Pie charts illustrating MEC values (µg/mL) and cellular responses to various product extracts. Segment size corresponds to the magnitude of biological effect, with larger segments indicating lower MECs, reflecting greater potency.

Impact of NAC Supplementation: Comparison of charts with and without NAC reveals that for 1R6F SbMed, oxidative stress is a major driver of biological effects. In contrast, NAC had minimal to no impact on the biological responses induced by 1R6F-TPM, Snus, or TFNPs.

p-c-JUN Signalling Dynamics: Orange segments represent p-c-JUN MECs, which showed opposing response directions for the smoke and smokeless test articles (see orange arrows).

Osmolarity-Linked Effects: As shown in Table 1, increasing concentrations of TFNP/Snus correlated with elevated osmolarity, which was associated with suppressed p-c-JUN signalling and enhanced apoptotic activity—consistent with findings by Xia *et al.* (2007).

3.2 Oral-Immune co-culture High Content Screening

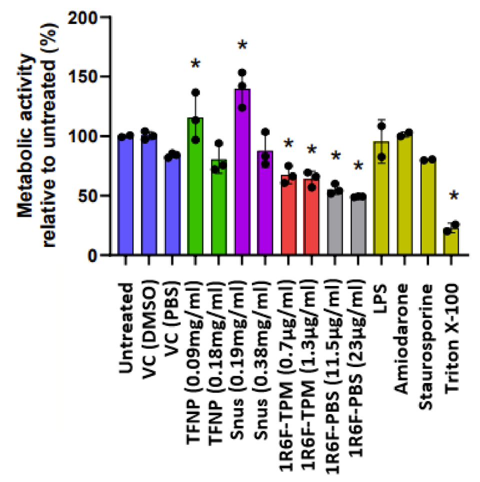
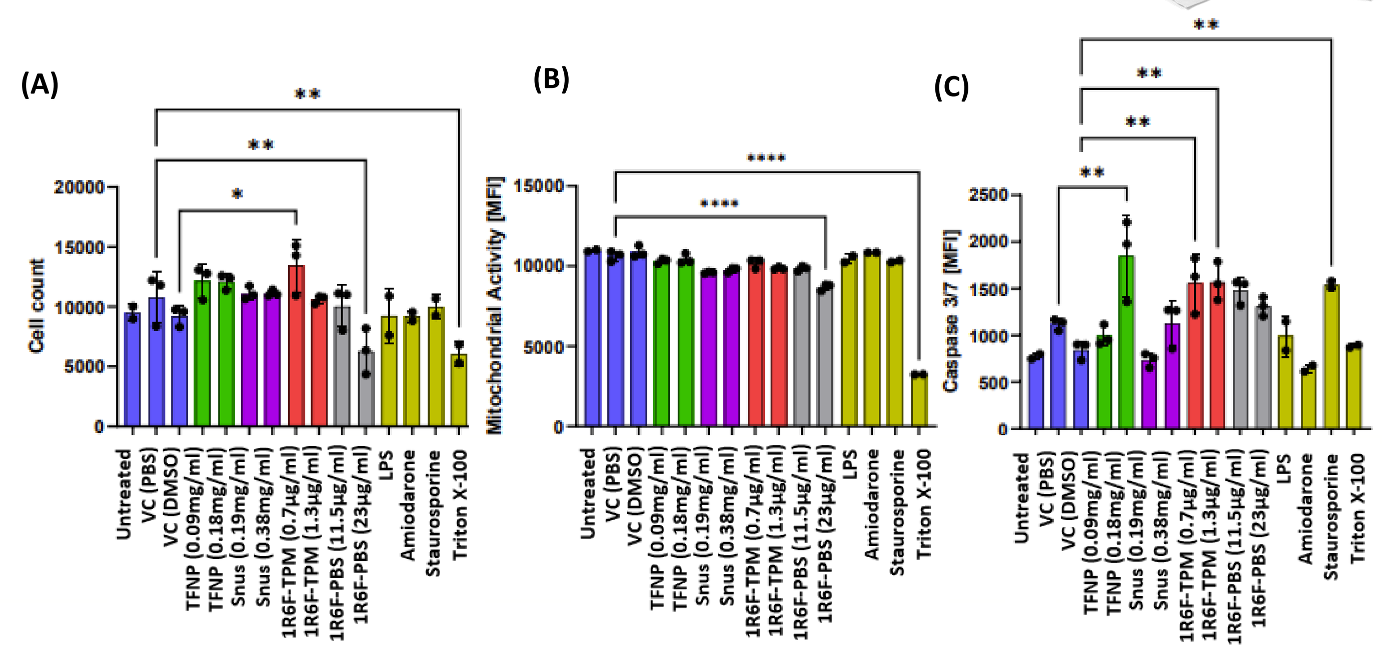


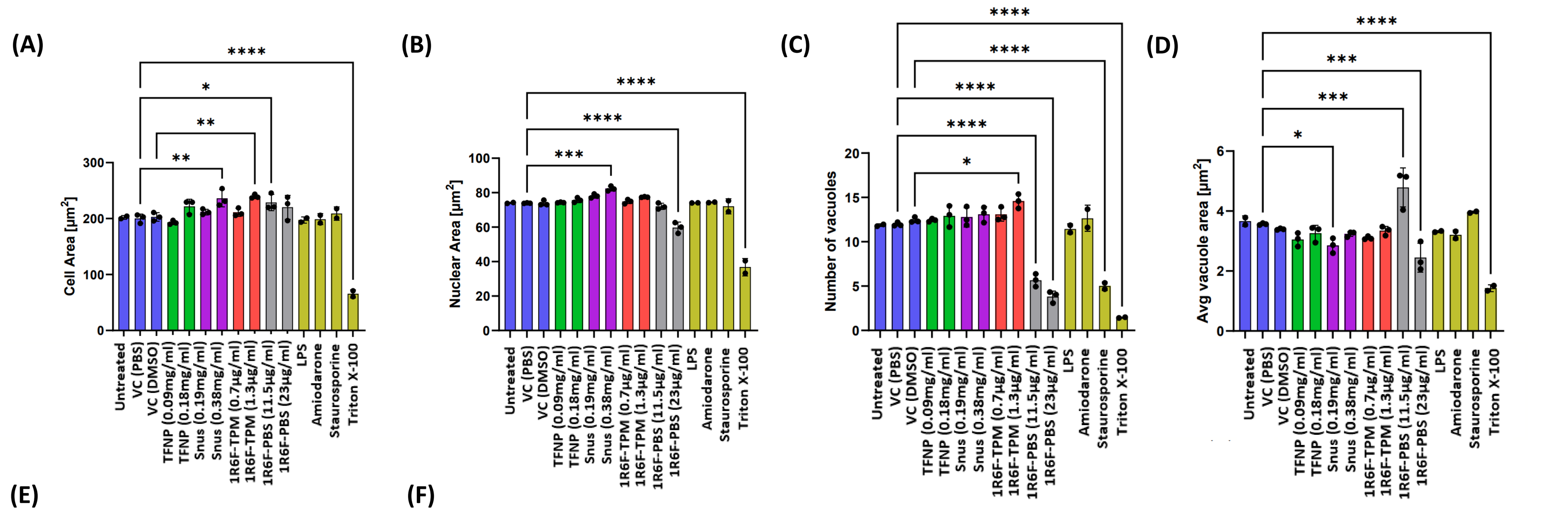
Figure 3: PrestoBlue activity after 48h treatment with test items and controls. Data are presented as a mean +/- SD of n=3 wells. Statistical significance was determined using one-way ANOVA with Dunnet's multiple comparison test and marked as *p<0.05.

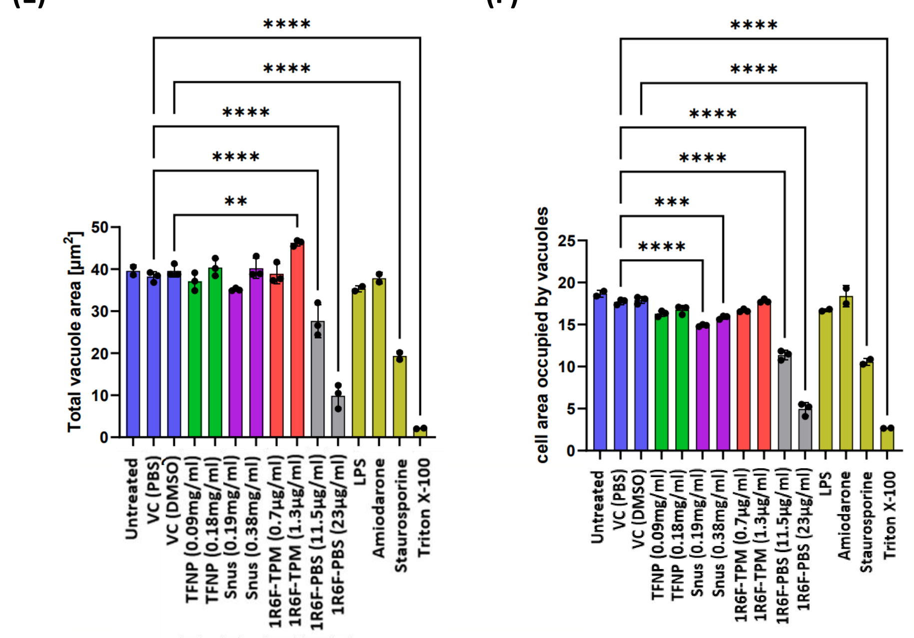
TFNP & Snus: Metabolic activity increased significantly with low-dose treatments. **1R6F-TPM/PBS:** both concentrations caused significant reductions in metabolic activity.



Figures 4A-C: Assessment of cell health after 48h exposure to test items and controls. Three cell health parameters were investigated: (A) cell count, (B) mitochondrial activity, and (C) early apoptosis. Data are presented as a mean +/- SD of at least 2 wells (with between 500 to 1500 cells per well analysed). Results are compared to the appropriate control and assessed for statistical significance, which was determined using One-way ANOVA with Dunnet's multiple comparison test. Statistical significance is marked as follows: *indicates p<0.05, **indicates p<0.01, ***indicates p<0.001 and **** indicates p<0.0001.

TFNP: High exposure treatment (0.18 mg/mL nicotine) significantly increased caspase 3/7 activity). **Snus:** No significant effects on cell health. **1R6F-TPM:** Caspase 3/7 activity increased at both concentrations (p<0.01). **1R6F-PBS:** High exposure treatment (23 µg/mL nicotine) reduced cell count (p<0.01) and mitochondrial activity (p<0.0001).





Figures 5A-F: Assessment of cell morphology after 48h exposure to test items and controls. Six morphological parameters were investigated: (A) cell area, (B) nuclear area, (C) number of vacuoles per cell, (D) percent of cell area occupied by total number of vacuoles, (E) area of a single vacuole per cell, and (F) total cell area occupied by vacuoles. Data are presented as a mean +/- SD of at least 2 wells (with between 500 to 1500 cells per well analysed). Results are compared to the appropriate control and assessed for statistical significance, which was determined using One-way ANOVA with Dunnet's multiple comparison test. Statistical significance is marked as follows: *indicates p<0.05, **indicates p<0.01, ***indicates p<0.001 and **** indicates p<0.0001.

TFNP: There were no significant changes observed in cell morphology.
Snus: High exposure treatment (0.38 mg/mL nicotine) increased cell and nuclear area (p<0.01, p<0.001) and reduced vacuole area and occupancy (p<0.05).
1R6F-TPM: High exposure treatment (1.3 µg/mL nicotine) increased cell area (p<0.01), vacuole number (p<0.05), and total vacuole area (p<0.01).
1R6F-PBS: Low exposure treatment (11.5 µg/mL) increased cell area (p<0.05), while high exposures reduced nuclear area (p < 0.0001). Both concentrations resulted in significantly reduced number of vacuoles per cell (p < 0.0001), that occupied a lower percentage of the cell (p < 0.0001).

CONCLUSIONS

- Under the conditions of the NAMs utilised in this study, cigarette smoke extracts induced strong, dose-dependent changes in apoptosis, DNA damage, oxidative stress, and macrophage morphology at concentrations as low as 0.7 µg/mL nicotine. In contrast, snus and TFNP extracts elicited responses only at concentrations more than 100-fold higher than those of cigarette smoke extracts, demonstrating a marked reduction in potency. Notably, TFNP extracts did not induce any significant morphological changes in macrophages within the oral-immune coculture system, whereas the other test articles did.
- Oxidative stress was identified as a major contributor to the biological effects of 1R6F SbMed in HCAECs. In contrast, the effects observed in HCAECs exposed to TFNP and snus were most likely attributable to increased osmolarity rather than specific toxicological mechanisms. Further investigations using a broader range of test item concentrations, osmolality measurements, and NAC supplementation are required in the oral-immune coculture system to better understand product-specific effects and potential mechanisms.
- Overall, the results from these two innovative tests, show that TFNPs have the potential to offer a harm reduction alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction.

REFERENCES

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