NEXT GENERATION PRODUCTS

High content screening of tobacco free nicotine pouch extracts shows marked reductions in toxicity when compared to cigarette smoke extracts

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INTRODUCTION

Tobacco-free nicotine pouches (TFNPs) are an emerging category of oral nicotine products increasingly used as an alternative to traditional tobacco products. However, their potential biological effects remain poorly characterised, especially in comparison to conventional cigarettes and tobacco-containing products like snus. To address this gap, we applied High Content Screening (HCS) - a sensitive, multiparametric *in vitro* method - to assess cellular responses in human coronary artery endothelial cells (HCAECs).

HCS enables detailed phenotypic profiling of stress, oxidative damage, and inflammation at the single-cell level, offering greater sensitivity than conventional assays recommended by CORESTA. We evaluated aqueous extracts from TFNPs, snus, and 1R6F reference cigarette smoke (smoke-bubbled medium [SbMed] and DMSO extracts of total particulate matter [TPM]) after 4 and 24 hours of exposure, with and without the reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC).

Markers of cellular stress (p-c-JUN), oxidative stress (SRXN1), DNA damage (γH2AX), and inflammation (NFκB translocation) were assessed via antibody staining, alongside nuclear size, glutathione (GSH) levels, and apoptosis (caspase 3/7 activity). Minimum effective concentrations (MECs) on a nicotine basis revealed substantially higher biological activity for 1R6F TPM and SbMed compared to snus and TFNPs. NAC co-treatment notably reduced SbMed-induced responses, indicating the strong oxidative stress potential of water-soluble gas-vapour phase (GVP) components in oxidative stress induction.

These findings support the utility of HCS for evaluating emerging nicotine products and suggest a lower biological activity profile for TFNPs relative to cigarette smoke.

These results underscore the significantly lower biological activity and oxidative stress potential of snus and TFNP extracts compared to cigarette smoke extracts, supporting their potential as a reduced-harm nicotine delivery alternative.

METHODS

Snus/TFNP Extract Preparation

Extracts from snus and tobacco-free nicotine product (TFNP) were generated following the method outlined by Yu et al. (2022), substituting PBS with EGM-MV2 medium to enhance exposure potential. Nicotine content was quantified, and extracts were stored at −70 ± 6 °C.

TPM and SbMed Extracts

1R6F reference cigarettes were smoked under Health Canada Intense conditions. Total particulate matter (TPM) was collected on Cambridge filter pads and extracted with DMSO. For SbMed extracts, smoke was bubbled through cell culture medium (adapted from Czekala et al., 2020), with quantification of nicotine and 8 carbonyls. Extracts were frozen at −70 ± 6 °C.

Cell Culture and Exposure

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 1.2×10^5 cells/mL). After overnight adherence, cells were exposed for 4 or 24 h with or without 1mM NAC (upper vs. lower plate rows). Extract concentrations and osmolarities are detailed in Table 1. Pure nicotine (20–1500 µg/mL) was used to assess nicotine-specific effects.

Immunostaining

Post-exposure, cells were fixed (4% formaldehyde), permeabilised (0.1% Triton X-100), and blocked in PBS/BSA. Primary antibodies (1:250–1:500) targeted p-c-JUN, SRXN1, NFκB, and γH2AX. Fluorophore-conjugated secondary antibodies (640 nm and 740 nm) minimised autofluorescence. Nuclei were counterstained with DAPI. Imaging and quantification were performed using the X-finity High Content Screener (Thermo).

GSH and Caspase 3/7 Assays

Caspase 3/7 activity was assessed using CellEvent Green Detection Reagent (C-10423). Intracellular GSH was stained with monochlorobimane (mBCL). Hoechst 33342 was used for nuclear counterstaining. Fluorescence was measured post-fixation on the High Content Screener.

Data Analysis

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. Nicotine itself was dosed from 20 to 1500µg/ml (not shown in the table). As separate measure the osmolarity values are added [osm/L]. The results indicate a high osmolar activity of the snus/ TFNP samples.

RESULTS

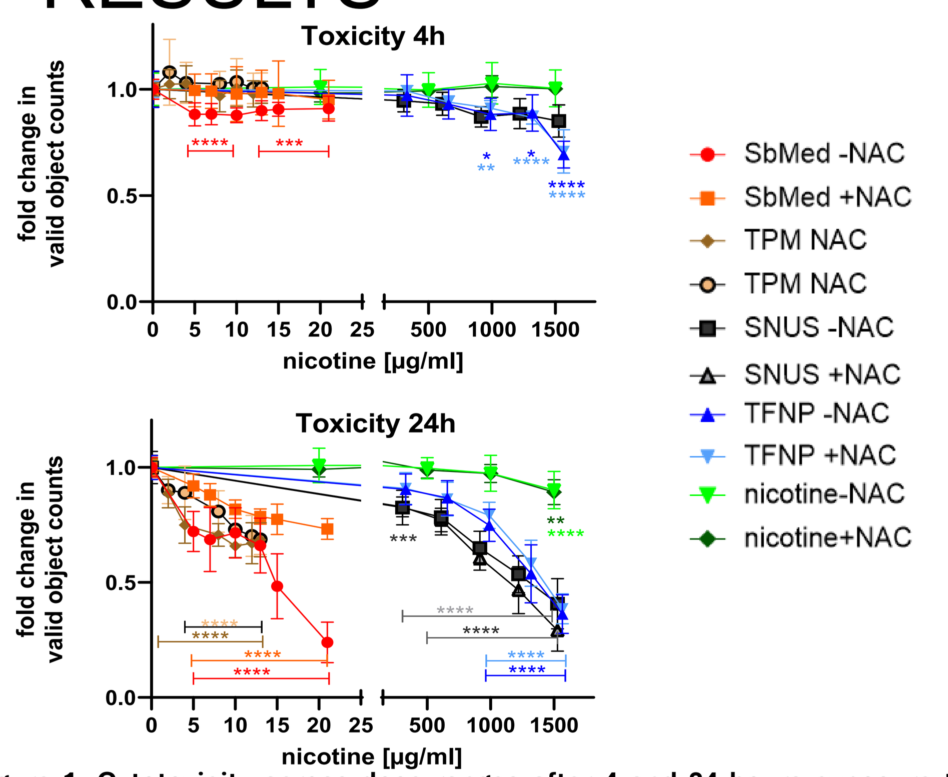

Figure 1. Cytotoxicity across dose ranges after 4 and 24 hours exposure to various extracts, with and without NAC.
Cell counts were used to assess cytotoxicity. NAC presence during SbMed exposure showed the strongest inhibitory effect, indicating high oxidative stress potential. Toxicity from snus and TFNP was mainly linked to apoptosis via elevated osmolarity. Nicotine caused only minor effects, regardless of NAC.
Key finding:
SbMed + NAC → strongest cytotoxicity, suggesting oxidative stress involvement
*Significance: *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001.

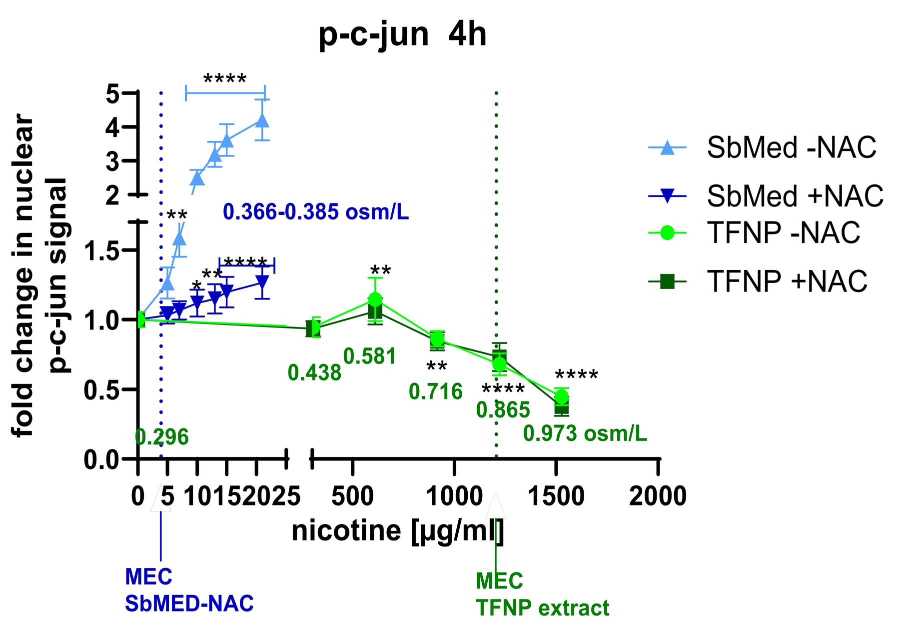

Figure 2. Inverted cellular stress response (p-c-JUN) in cells treated with TFNP vs. SbMed extracts.
SbMed triggered a sharp increase in p-c-JUN fluorescence, strongly inhibited by NAC. In contrast, TFNP reduced signal intensity, with no NAC effect. Increasing osmolarity (numerical labels) correlated with reduced p-c-JUN and apoptosis, consistent with Xia et al. (2007).
Key finding:
SbMed → strong p-c-JUN activation (oxidative stress); TFNP → signal suppression via osmotic stress
*Significance: *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001.

RESULTS (continued)

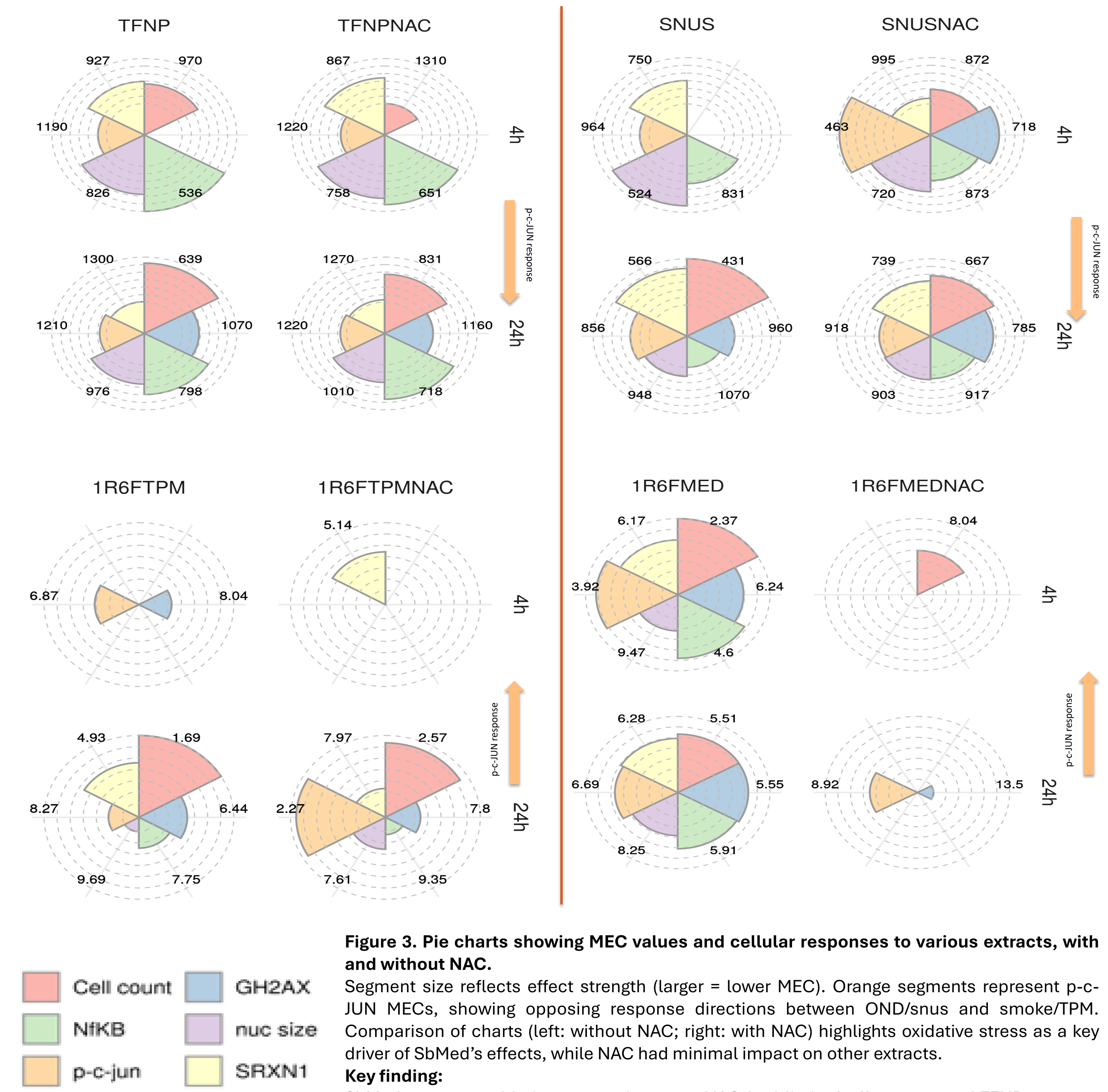

Figure 3. Pie charts showing MEC values and cellular responses to various extracts, with and without NAC.
Segment size reflects effect strength (larger = lower MEC). Orange segments represent p-c-JUN MECs, showing opposing response directions between OND/snus and smoke/TPM. Comparison of charts (left: without NAC; right: with NAC) highlights oxidative stress as a key driver of SbMed's effects, while NAC had minimal impact on other extracts.
Key finding:
SbMed → strong oxidative stress signature; NAC had limited effect on snus/ TFNP extracts. When detected effects induced by the latter were associated to very high MEC values.

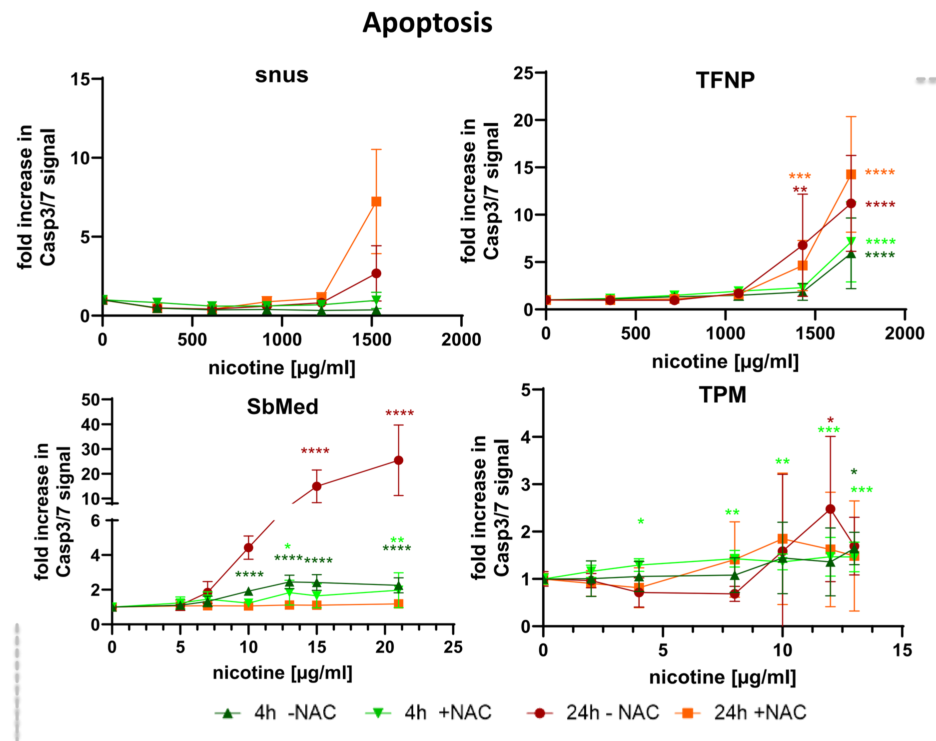

Figure 4. Apoptosis induction in HCAECs after 4h and 24h exposure to various extracts, with and without NAC.
Caspase 3/7 activation was measured via green fluorescence (CellEvent kit), corrected for autofluorescence. snus and TFNP induced apoptosis at doses with elevated osmolarity and reduced nuclear size. SbMed-induced apoptosis was mainly oxidative stress-dependent, especially after 24h. TPM showed a modest increase, unaffected by NAC.

Key finding:

SbMed → apoptosis driven by oxidative stress; TFNP/snus → apoptosis linked to osmotic stress
*Significance: *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001.
Legend applies to all four diagrams.

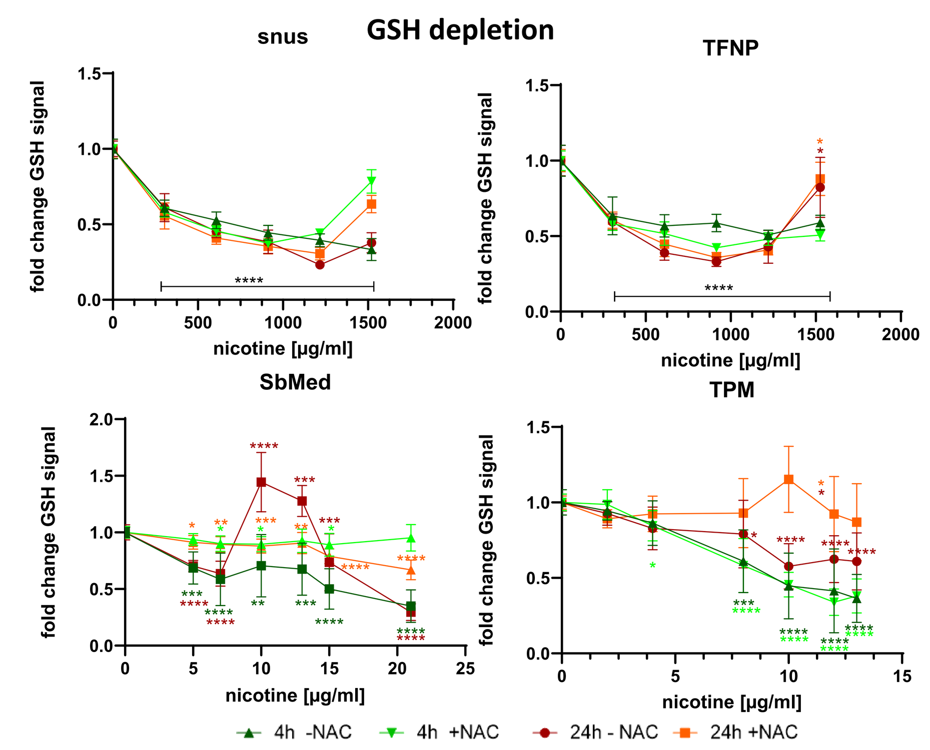

Figure 5. GSH depletion in HCAECs after 4h and 24h exposure to tobacco-related extracts.
Dose-response curves show GSH levels following exposure. Snus and TFNP caused significant GSH reduction, mostly NAC-independent. SbMed led to strong depletion, with high NAC responsiveness at 4h. At ~30–40% toxicity, cells overcompensated by increasing GSH above baseline. TPM showed delayed regulation, with NAC enhancing compensation at 24h.

Key finding:

SbMed → strong GSH depletion with NAC responsiveness; TFNP/snus → NAC-independent depletion
*Significance: *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001. *Legend applies to all four diagrams*.

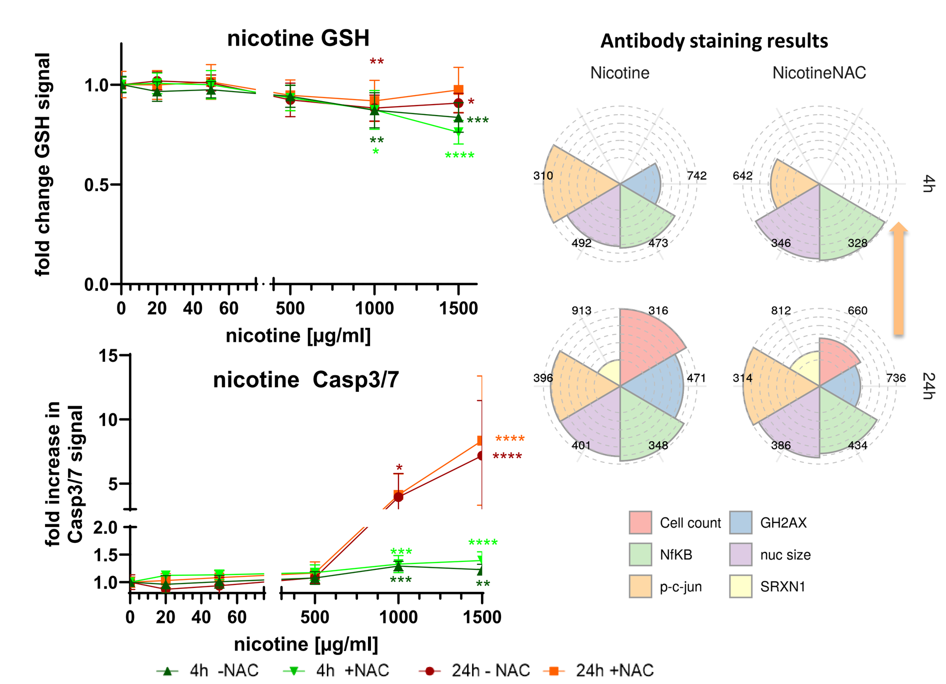

Figure 6. GSH depletion, apoptosis, and immunostaining in HCAECs after nicotine exposure (4h and 24h), with and without NAC.
Nicotine caused moderate GSH depletion at 4h, compensated by 24h; NAC had no effect. Apoptosis was significantly induced at both time points, though much stronger after 24hours but without NAC effect at any time point. Immunofluorescence showed no NAC-related differences. Cell count reduction and oxidative stress appeared only at 24h and all effects started at high MEC values.
Key finding:

Nicotine → apoptosis and mild oxidative stress, largely NAC-independent
*Significance: *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001.
Legend applies to both diagrams.

CONCLUSIONS

Under the test conditions, the cigarette smoke extract (1R6F reference cigarette) triggered clear oxidative stress responses, which were significantly reduced by the antioxidant N-acetylcysteine (NAC), a known reactive oxygen species (ROS) inhibitor. This suggests that water-soluble components in the gas-vapour phase (GVP) of cigarette smoke play a key role in driving oxidative stress.

In contrast, extracts from TFNP and snus showed minimal oxidative stress potential. The few biological responses observed occurred only at high nicotine concentrations and appeared unrelated to oxidative stress mechanisms. These effects are more likely due to increased osmolarity (see also Xia et al, 2007) in the extracts rather than specific toxicological pathways.

Importantly, the nicotine concentrations at which effects were seen were much higher than typical consumer exposure levels as shown by Azzopardi et al. (2025). This supports the potential of TFNP and snus products as reduced-harm alternatives to conventional cigarettes, which may contribute meaningfully to tobacco harm reduction.

REFERENCES

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