Comparison of heated tobacco product aerosol to cigarette smoke in human bronchial epithelial tissues using High Content Screening

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1. INTRODUCTION

Laboratory studies have shown heated tobacco product (HTP) aerosols have substantially reduced numbers and levels of harmful and potentially harmful constituents relative to combustible cigarette smoke. The biological impact of these aerosols needs to be determined to assess their potential impact on consumer health. In the present study we utilised High Content Screening (HCS) on normal human bronchial epithelial (NHBE) cells to measure the biological impact of a range of HTPs (n=6, 3 different variants per device), compared to the reference cigarette (1R6F). HCS enables the examination of multiple cell endpoints in the same cell, at the same time using different florescent dyes or Immunofluorescence stains, to stain multiple epitopes at the same time.

2. MATERIALS AND METHODS

HTP aerosols and cigarette smoke were bubbled through a series of 3 impingers each containing 10ml of PBS to capture components of the smoke / aerosol. Both product categories were puffed in accordance with ISO 20778 (55ml, 30sec interval, 2 sec puff duration), yielding aqueous extracts concentrations of 1.8 puffs/ml for reference cigarettes and 4.8 puffs /ml for HTPs using a Vitrocell VC-10S Type smoking robot. Positive controls used are summarised in Table 1. Trapped nicotine and carbonyls were quantified within the aerosol and smoke bPBS samples (See Table 2). All samples were stored frozen at −70 °C prior to analysis. Nicotine was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, USA) using nicotine-d4 as the internal standard. For the analysis of Carbonyls, bPBS samples were diluted with 2,4-dinitrophenylhydrazine (DNPH). The carbonyl-DNPH derivates, were then quantified using high performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent Technologies 1100 Series).

NHBEs (donor: 65 year old/ male/ Caucasian/ non-smoker) were obtained from PromoCell GmbH; and maintained at 37°C in an atmosphere of 5% CO_2 in Airway Epithelial Cell Growth Medium (AEGM). Cells were treated with increasing concentrations of PBS ranging between 0 – 10% varying between test articles depending on specific toxicity. NHBE cells were treated for 4 hours (2 hours for glutathione content) and 24 hours with the different extracts and were subsequently stained with antibodies/ dyes to investigate the impact on 6 endpoints (5 for 24 hours) (Cell count, glutathione content (oxidative stress), ɣ-H2AX phosphorylation (double DNA strand breaks), c-jun phosphorylation (cell stress), cytochrome C release (Apoptosis) and NFkB translocation (inflammatory and other pathways) by means of HCS technology. The direction of change for the different HCS endpoints is summarised in Table 1. HCS images are shown in Figure 1.

Table 1: HCS metrics for different parameters and positive controls used.

Assay	Biological end point	Cellular compartment	Out put feature	Direction of adverse change	Positive control
Cell count	Cytotoxicity	Nucleus	Cell count	decrease	none
Cytochrome C	Apoptosis	Cytoplasm/nucleus	Average ring intensity	decrease	Staurosporine
Gamma H2AX	DNA double strand breaks	Nucleus	Average circle intensity	Increase	Etoposide
Phospho- cJun	Cellular stress	Nucleus	Average circle intensity	Increase	Staurosporine 2 hours; etoposide 24 hrs
NFkB	Inflammation	Nucleus/cytoplasm	Circ/ring average intensity difference	increase	TNF alpha
GSH depletion	Oxidative stress	Cytoplasm	Circle whole cell	decrease	H_2O_2

Table 1 summarises the change of direction expected (either increased or decreased) and the respective positive controls used for each HCS endpoint.

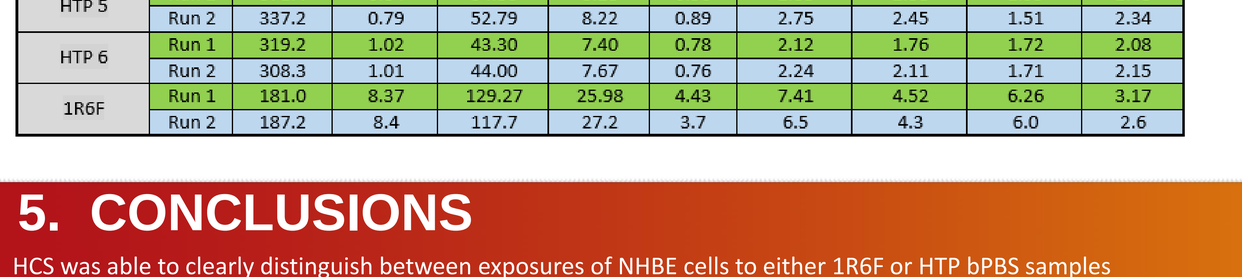


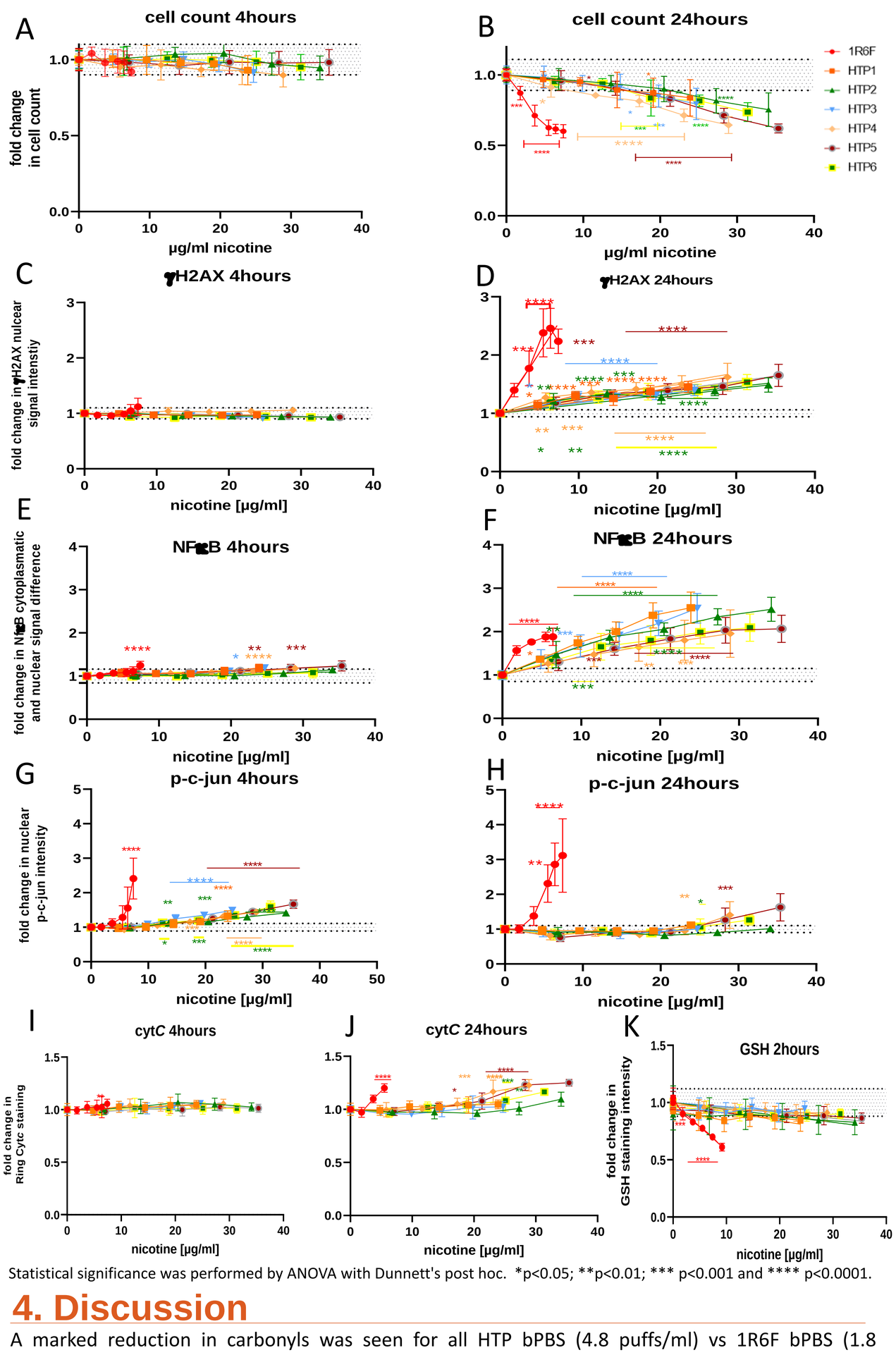
Figure 1: Example of HCS images for untreated NHBE cells partitioned into circle and ring, depending on if the effects are seen in the whole cell or located in specific regions such as the nucleus. The cells are evaluated for the red and green fluorescence intensity indicating the level of γH2AX (circle regions; red) and amount of NfκB respectively (circle and ring regions, green), in the combined coloured image (far right).

3. Results

Table 2: Nicotine and carbonyls measured in trapped PBS samples

Test article	Run No.	Nicotine [µg/mL]	Form aldehyde [µg/mL]	Acet aldehyde [µg/mL]	Acetone [µg/mL]	Acrolein [µg/mL]	Propion aldehyde [µg/mL]	Croton aldehyde [µg/mL]	2-Butanon (MEK) [µg/mL]	n-Butyr aldehyde [µg/mL]
HTP 1	Run 1	270.9	1.03	45.80	5.26	0.57	2.32	1.11	0.89	1.85
	Run 2	207.8	0.97	44.05	5.26	0.56	2.21	1.15	0.86	1.84
HTP 2	Run 1	383.6	1.45	52.34	7.25	0.73	2.67	1.32	1.27	1.98
	Run 2	298.7	1.2	51.8	7.0	0.8	2.7	1.3	1.4	2.1
HTP 3	Run 1	251.7	1.24	48.00	6.91	0.74	2.48	1.23	1.24	1.92
	Run 2	242.4	1.3	46.2	6.5	0.7	2.3	1.2	1.2	1.8
HTP 4	Run 1	310.8	0.87	59.11	7.09	1.06	3.12	1.69	1.78	2.68
	Run 2	266.7	0.86	57.66	7.11	0.95	3.00	2.02	1.72	2.59
HTP 5	Run 1	371.4	0.84	54.30	8.28	0.96	2.88	2.38	1.59	2.43
	Run 2	337.2	0.79	52.79	8.22	0.89	2.75	2.45	1.51	2.34
HTP 6	Run 1	319.2	1.02	43.30	7.40	0.78	2.12	1.76	1.72	2.08
	Run 2	308.3	1.01	44.00	7.67	0.76	2.24	2.11	1.71	2.15
1R6F	Run 1	181.0	8.37	129.27	25.98	4.43	7.41	4.52	6.26	3.17
	Run 2	187.2	8.4	117.7	27.2	3.7	6.5	4.3	6.0	2.6

Figure 2 (A-K): show the data for fold change for each HCS endpoint for HTP vs 1R6F relative to the nicotine concentration.



Statistical significance was performed by ANOVA with Dunnett's post hoc. *p<0.05; **p<0.01; *** p<0.001 and **** p<0.0001.

4. Discussion

A marked reduction in carbonyls was seen for all HTP bPBS (4.8 puffs/ml) vs 1R6F bPBS (1.8 puffs/ml). The 1R6F reference cigarette bubbled PBS, caused a significant dose dependent decrease in cell count and significantly altered y-H2AX, NfKB, p-c-Jun and glutathione endpoints (at concentrations >0.2 µg/ml nicotine). A partial overlap with endpoints induced by the HTP solutions was also observed but at concentrations considerably higher (>1.61 µg/ml nicotine). The fold change difference in responses as measured by minimum effective concentrations was dependent upon the end point being measured but was between 1.89-10.38 fold higher concentrations for the HTP when compared to the 1R6F on a nicotine basis.

For all of the HTP products there was no changes with cytochrome c at 4 and 24 hours up to the highest nicotine concentrations tested (35 µg/ml), the reason for this will need further investigation. As expected for the majority of the remaining endpoints larger differences between HTP and 1R6F were seen at 24 hours.

5. CONCLUSIONS

- HCS was able to clearly distinguish between exposures of NHBE cells to either 1R6F or HTP bPBS samples
- There was a significant reduction in toxicity of both HTP devices and across multiple stick variants when compared to 1R6F
- The two different HTP devices were similar in terms of response for the individual HCS endpoints as seen by the overlapping error bars.
- These results add to the growing body of evidence that HTPs are likely to be considerably less harmful than combustible cigarettes and offer significant harm reduction potential for those adult smokers that choose to transition to these products as an alternative to continued smoking.

REFERENCES

1. ISO 20778: 2018 cigarette- Routine analytical cigarette smoking machine – definitions and standard conditions with an intense smoking regime https://www.iso.org/standard/69065.html
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