Next Generation Product (NGP) Aerosols Induce Lower Biological Activity Than Combusted Cigarettes: A Comparison of Aerosol Bubbled Extract Chemistry and *in Vitro* Toxicity

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1. INTRODUCTION / OBJECTIVES

With the increased popularity of next generation aerosol products (NGP) it is important that their potential biological effects can be assessed quickly and accurately. Exposure of these products to *in vitro* assays requires techniques which can deliver any biologically relevant compounds to the cellular system that are human physiologically relevant. Ideally, cells should be exposed to the whole smoke/aerosol, however not all cell techniques can be exposed in this manner. An alternative technique, is through the bubbling of cigarette smoke or NGP aerosol through a solution of Phosphate Buffered Saline (PBS).

The objective of this study was to determine the suitability of aerosol bubbled PBS as an aerosol exposure medium, to compare the biological activities of cigarette smoke and NGP aerosol in established *in vitro* toxicological assays.

2. MATERIALS AND METHODS

2.1 Test Samples (all Commercially available)

- 3R4F Kentucky Reference Cigarettes
- Tobacco Heated Product (THP)
- Hybrid Product (HYB); 1.8 % nicotine
- myblu™ Tobacco flavour; 1.6% nicotine

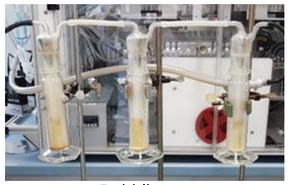


Figure 1 Bubbling smoke/vapour exposure system

2.2 Smoke / Aerosol Extract Generation Method

Aerosol from test products was generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line Impingers each containing 10 mL Phosphate Buffered Saline (See Figure 1). A total stock solution of 30mls per test article was used: 1.8 puffs per ml for 3R4F and 4 puffs per ml for NGPs.

Table 1: Smoke and Aerosol was generated using the following regimens:

Sample	Smoking Regime	Puff Volume (ml)	Puff Duration (Seconds)	Puff Interval (Seconds)	Vent Blocking	Puff Profile	Puff Count
3R4F Cigarette	Health Canada Intense	55	2	30	Yes	Bell Shaped	54
THP	Health Canada Intense	55	2	30	N/A	Bell Shaped	120
HYB	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	120
myblu	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	120

Nicotine and carbonyls trapped in fresh PBS samples were quantified using an LC-MS/MS and HPLC-DAD method respectively. For nicotine measurement, the internal standard Nicotine-d4 was used. For carbonyl determination DNPH (2,4-Dinitrophenylhydrazine) was used and the carbonyl-DNPH derivate were detected. The replicates per sample are summarised in Table 2.

2.3 In Vitro Toxicology

The neutral red assay (NRU) was conducted using HepG2 and BEAS2B cells following standard assay protocols in accordance with ISO 17025. BEAS-2B (ECACC; Cat.No.: 95102433) cells were grown in bronchial epithelial growth medium (BEGM; Lonza). HepG2 (ATCC, Cat.No.: HB8065) liver cells were grown in MEM & Weymouth's 705/1 (4:1) supplemented with Ultroser-G.

The Ames assay was conducted in accordance with OECD (471) method using strains TA98 and TA100. The In Vitro Micronucleus (IVM) assay was conducted with V79 cells (3 hrs +/- S9; 24 hrs –S9) to OECD 478. The in vitro Cellular Transformation assay (CTA) assay (Bhas 42) was conducted by BioReliance using thawed samples according to the OECD draft Guidelines (2016).

3. RESULTS

3.1 Nicotine Quantification of PBS extracts

3R4F nicotine was trapped at the highest concentrations in the second impinger, all three were combined provide 30 mls for testing. Nicotine delivery was highest for myblu™ (152.0 µg/ml) and lowest for HYB (53.0 µg/ml) .

Figure 1: Trapping of nicotine in each impinger (Imp) with 10 mls PBS (n=19); Error bars show the standard deviation. Variation Coefficient Imp 1 26%, Imp 2 30%, Imp3 42%.

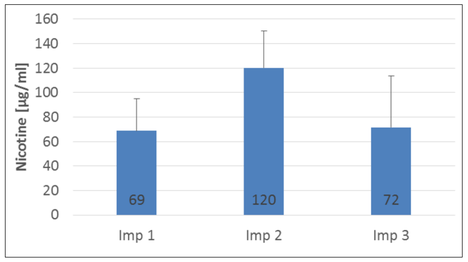


Table 2: Nicotine quantification in combined PBS samples; mean values of corresponding replicates.

Sample	Nicotine [µg/ml]	Replicates (n)
3R4F	82.5	23
THP	123.0	10
HYB	53.0	7
myblu™	152.0	9

3.2 Carbonyl Quantification of PBS Extracts

As expected carbonyl levels were the highest in 3R4F and THP. Limited carbonyls were detected in HYB extracts and none in myblu™ extracts.

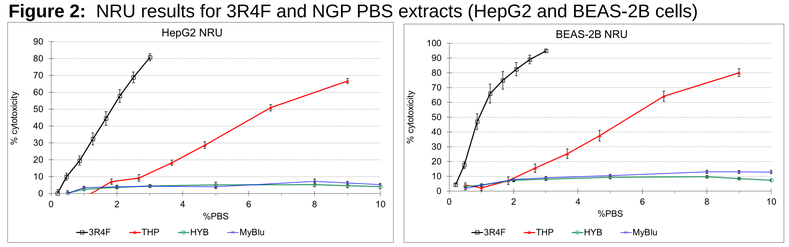
Table 3: Carbonyls quantification in pooled PBS samples

Carbonyl levels (µg/ml)	3R4F	THP	HYB	myblu™	LOQ
Formaldehyde	5.9	0.9	1.0	<LOQ	0.25
Acetaldehyde	157.1	52.9	<LOQ	<LOQ	1.5
Acetone	24.0	5.4	<LOQ	<LOQ	1.0
Acrolein	9.4	1.3	0.5	<LOQ	0.5
Propionaldehyde	9.5	3.5	<LOQ	<LOQ	0.5
Crotonaldehyde	6.2	0.6	<LOQ	<LOQ	0.5
2- Butanone (MEK)	6.3	1.3	<LOQ	<LOQ	0.5
n-Butyraldehyde	3.6	2.8	<LOQ	<LOQ	0.5

LOQ = limit of quantification

3.3 Cytotoxicity of PBS extracts (NRU)

Figure 2: NRU results for 3R4F and NGP PBS extracts (HepG2 and BEAS-2B cells)

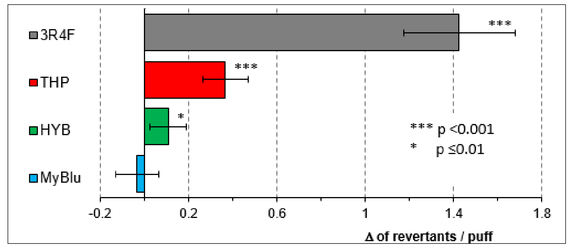


After exposure (65 ± 4 hours) with up to 10% of PBS extracts neutral red staining /extraction was applied. There was a clear effect of cytotoxicity for 3R4F with a reduced effect for THP and none for myblu™ and HYB under the conditions of test (n=3).

3.4 Mutagenicity of PBS extracts

There was marked mutagenicity with 3R4F in PBS with TA100+S9 which was reduced for THP (approx. 3 times lower), weak for HYB and no effects were observed for myblu™ (n=3) under the conditions of test. With TA98+S9 (Data not shown) there was a mutagenic response with 3R4F smoke only.

Figure 3: Mutagenicity of PBS extracts with TA 100 (+S9)

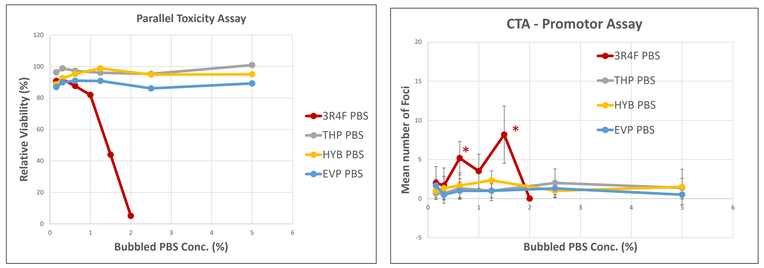


3.5 Genotoxicity (IVM) of PBS extracts

None of the PBS samples elicited any effects in the IVM assay

3.6 Cellular Transformation Assay of PBS extracts

Figure 4: Cytotoxicity and promotion activity of PBS extracts in Bhas 42 CTA assay.



3R4F showed extensive cytotoxicity at concentrations >1%. Weak to no cytotoxicity was observed for THP at the highest concentration (5%). For HYB and myblu™ there was no effect on cytotoxicity.

Only 3R4F was positive for Promoting activity with no bioactivity observed for any of the other NGP samples (* p<0.05 (ANOVA, Dunnett's post-hoc); statistically significant increase)

4. CONCLUSIONS

- This study demonstrates the suitability of aerosol bubbled PBS as an exposure media in established *in vitro* assays
- As expected, clear cytotoxicity and mutagenic effects were observed for 3R4F. Effects with THP were also positive, however, to a much lesser degree. No effects were observed for HYB or myblu™ samples under the conditions of test.
- Only 3R4F exhibited a tumor promotion response in the CTA assay.
- Using a core battery of *in vitro* tests, myblu demonstrated the lowest response compared to 3R4F, HYB and THP.
- Overall, the data shows the potential of these products, e-vapour products as less harmful alternatives to cigarettes.

REFERENCES

[1] OECD (2016) Guidance document on the in vitro Bhas42 Cell Transformation Assay, Series on Testing & Assessment No. 231
[2] Health Canada. Health Canada method T-115, determination of "tar", nicotine and carbon monoxide in mainstream tobacco smoke. (1999): http://edge.rit.edu/edge/P10056/public/Health%20Canada%20Nicotine
[3] Coresta Recommended Method Nº 81 https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf

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