Chemical and biological assessment of a Heated Herbal Product reveals marked reductions in aerosol toxicants and *in vitro* toxicity compared to cigarette smoke

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1. INTRODUCTION

Heated Herbal Products (HHPs) are an emerging category of potentially reduced harm nicotine products for adult smokers who would otherwise continue smoking.
The aim of the present study was to compare the chemical composition and *in vitro* toxicological activity of the aerosol of a novel HHP (Pulze 2.0 and iSenzia sticks) and cigarette smoke (1R6F Kentucky reference cigarette). iSenzia is an herbal stick containing nicotine extract which is heated using the PULZE device. The iSenzia sticks contain a tea-based substrate, rather than tobacco, and nicotine is added along with oolong and green tea, cellulose, binder, and different flavouring ingredients.

To assess the biological activity of the products, the HHP aerosol and 1R6F whole smoke were assessed using the CORESTA regulatory *in vitro* toxicology assays: neutral red uptake (BEAS-2B) for cytotoxicity, Ames (TA98, TA100; +/-S9) for mutagenicity and *in vitro* micronucleus assay (V79; +/-S9) for genotoxicity. The Smoke Chemistry compared the levels of the WHO TobReg9 analytes, nicotine, and ACM of the HHP aerosol with conventional cigarette smoke on a per puff basis.

2. METHODS

Test Articles
- 1R6F Reference Cigarette (University of Kentucky)
- Heated Herbal Product (HHP), (Pulze 2.0 and iSenzia Strong Menthol stick) (Imperial Brands PLC).

Figure 1. Pulze device and iSenzia stick configuration.

The Pulze device is an electrically powered heating device which is designed to heat the herbal portion of the iSenzia stick during consumer use to generate a nicotine-containing aerosol.

The iSenzia stick construction: filter cotton, capsule, capsule positioning paper rolls and tea-based substrate.

Smoke/Aerosol Generation

For the NRU and IVM assays, the fresh smoke/aerosol was generated using the Smoke Aerosol Exposure *In Vitro* System (SAEIVS; Burghart Tabaktechnik, Wedel, Germany). Generation of both cigarette smoke and aerosol was performed in basic accordance with the ISO 20778:2018 smoking regime (55ml puff volume / 2 sec. puff duration / 30 sec. puff interval / bell shape puff profile).

In the case of the Ames assay, the bacteria were exposed to the whole smoke/aerosol achieved using a Smoking Robot VC 10 S-Type (Vitrocell Systems GmbH, Germany). Generation of fresh smoke from reference cigarette 1R6F was performed according to ISO3308:2012 smoking conditions (35ml puff volume/ 2sec. puff duration/ 60sec. interval/ bell shape puff profile) while for the HHP ISO 20778:2018 without vent block was used to generate the aerosol.

Before the smoking procedure, the reference cigarettes were conditioned according to ISO 3402:2023, while the HHP sticks were not conditioned.

***In Vitro* Toxicology**
The following *in vitro* toxicological assays were performed:
- Mutagenic potential was determined using the *in vitro* Bacterial Reverse Mutation Test (Ames test) with *Salmonella* Typhimurium strains TA98 and TA100 (+/-S9) in compliance with the OECD Test Guideline 471.
- Cytotoxicity was determined using the *in vitro* Neutral Red Uptake (NRU) assay with the human bronchial epithelial cell line, BEAS-2B. Cells were exposed to increasing numbers of puffs (dose levels) of fresh whole aerosol at the air liquid interface (ALI).
- Genotoxicity was determined using the *in vitro* micronucleus test (IVM) with V79 hamster lung fibroblasts. V79 cells were exposed to increasing dose levels of fresh aerosol at the air liquid interface (ALI) followed by a subsequent short-term incubation with S9 mix (+S9) or by direct recovery following exposure (-S9).

Aerosol chemistry
- HHP products were vaped on a linear smoking machine (LM4C Borgwaldt, Hamburg, Germany) and the aerosol was trapped on either a Cambridge filter pad in a gas collection bag (e.g. Tedlar bag) or in impinger using DNPH solution depending on the targeted analytes.
- Aerosol collected mass (ACM) was trapped on a Cambridge filter pad. The mass of the filter pad including the holder of the smoking machine was determined before and after use. The mass of the collected particulate phase per stick is the aerosol collected mass (ACM).
- Tobacco Specific N-nitrosamines (TSNAs) - the collected ACM is extracted from the Cambridge filter pad with water/methanol. Individual NNN, NAT, NAB, NNK are quantified by LCMSMS using respective internal standards deuterated.
- Gas phase - the vapour phase of the aerosol was collected in a Tedlar bag located after the Cambridge filter. The sample (vapour phase) was separated by gas chromatography (GC) and 20 selected gas phase compounds are quantified by mass spectrometry (MS). Identification of the single components was carried out using retention time and their specific masses.
- Nicotine – the particle phase of the aerosol of the HHP was trapped on a Cambridge filter pad. The filter was extracted with Propan-2-ol. An aliquot is analysed via GC-Flame Ionisation Detector (FID).
- CO: The vapour phase of the aerosol is collected following a Cambridge filter pad. The carbon monoxide content is determined using an CO calibrated non-dispersive infrared analyser (NDIR). CO emission is calculated per stick.

3. RESULTS

1. *Smoke chemistry*

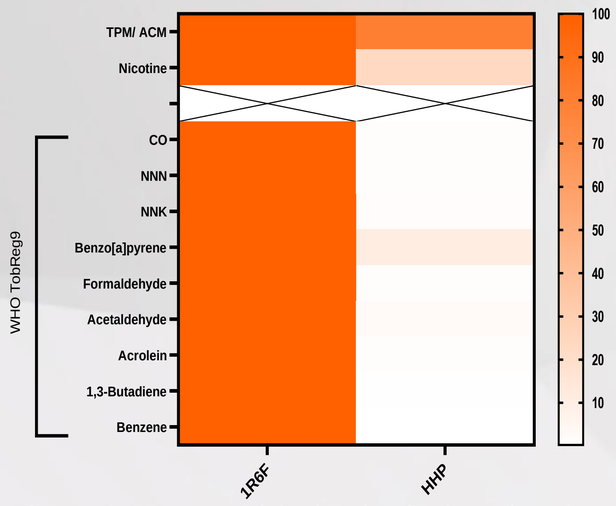

Figure 2. Heatmap of the levels of selected analytes in the 1R6F Reference Cigarette smoke and HHP stick aerosol, on a per puff basis, normalised to 1R6F values (100%). NNN = N-nitrosonornicotine; NNK = nicotine-derived nitrosamine ketone.

2. *Neutral Red Uptake assay*

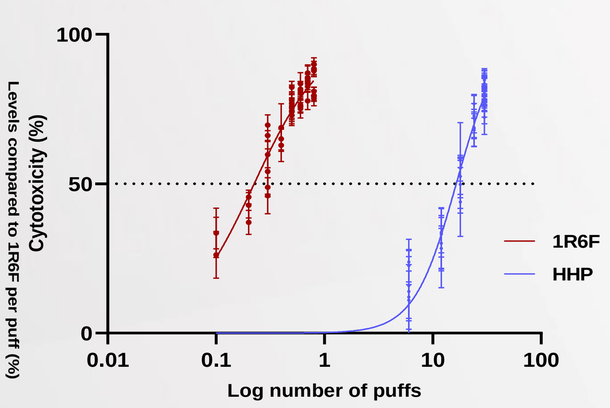

Figure 3: Percentage cytotoxicity induced in the neutral red uptake assay (Beas-2B cells) following exposure to increasing puff numbers (log scale) of 1R6F Reference Cigarette whole smoke, or HHP whole aerosol. Fifty percent cytotoxicity compared to negative control (EC_{50}) is marked with a black dotted line. Data shown are the mean +/- standard deviation (SD) with n = 3;

3. *Ames test*

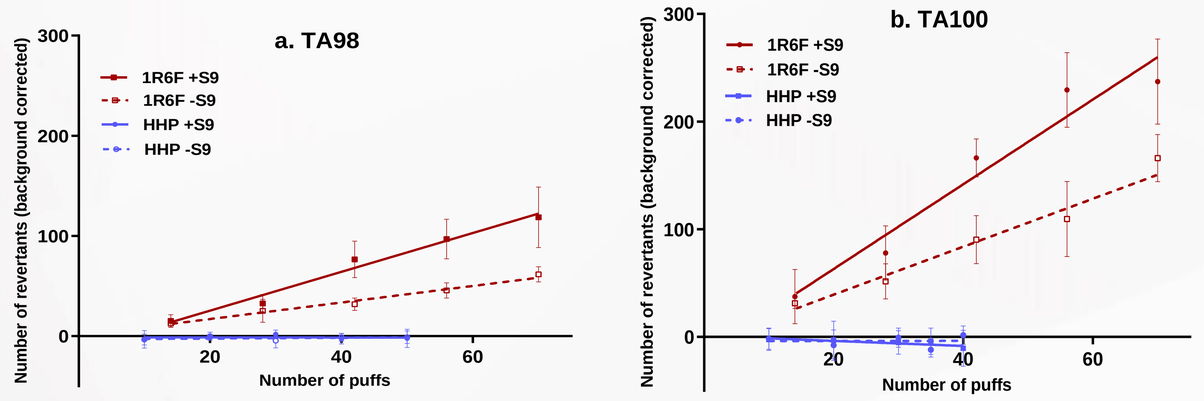

Figure 4: Average (background corrected) number of revertant colonies per plate for TA98 (a) or TA100 (b) strains exposed to increasing numbers of puffs of 1R6F Reference Cigarette smoke or HHP aerosol, +/-S9. n = 3; error bars represent SD. Linear regression analysis was applied to the data (trendlines as indicated) and the slope calculated.

4. *Micronucleus assay*

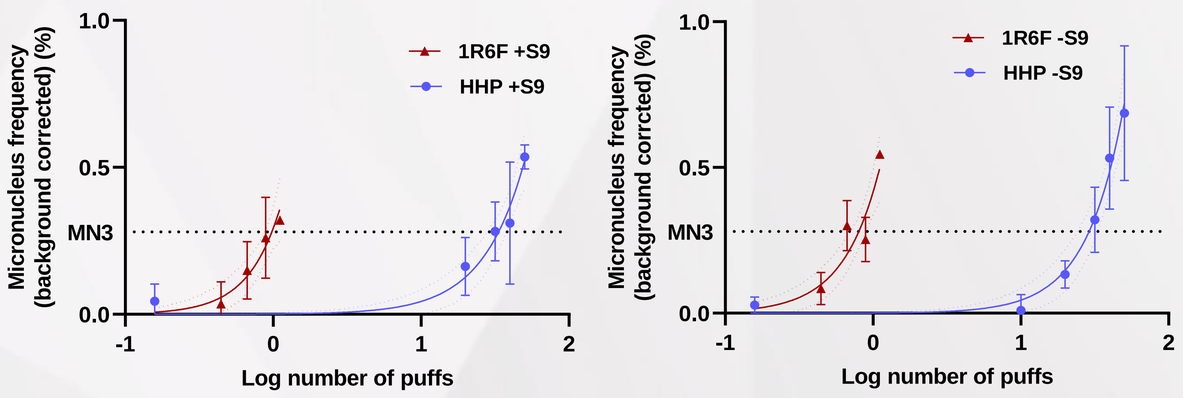

Figure 5: Background subtracted micronucleus frequency in V79 cells following exposure to increasing puffs (log scale) of 1R6F whole smoke or HHP whole aerosol in either the presence (a) or absence (b) of S9. ECMN3 (number of puffs necessary to reach the three-fold increase over background micronucleus frequencies) analysis was carried out using non-linear regression analysis (solid lines for each test item) to determine the puff-based concentration required to induce MN frequencies three-fold over background levels for each test article. Error bars represent SD; n = 2.

Chemical analysis of the HHP aerosol revealed substantial reductions (average 97%) in the WHO TobReg9 analytes when compared with conventional cigarette smoke on a per puff basis. The nicotine content was around 75%, and ACM 20%, less per puff compared to 1R6F.

The Neutral Red Uptake assay demonstrated marked reductions in HHP aerosol cytotoxicity compared to 1R6F Reference Cigarette.
- The data indicates that the aerosol from HHP was 73-fold less cytotoxic than smoke from the 1R6F Reference Cigarette based on EC50 comparison on a puff basis.

The HHP was not mutagenic, whereas 1R6F was mutagenic under the test conditions.
- Smoke generated from the 1R6F reference cigarette caused statistically significant (positive slope $p<0.05$) and reproducible increases in the number of revertants (+/-S9) mix.
- The HHP did not meet the criteria to be classified as mutagenic in TA98 and TA100 (+/-S9) under the conditions applied.

***The In Vitro* Micronucleus assay showed marked reductions in HHP aerosol genotoxicity compared to 1R6F Reference Cigarette smoke.**
- Dose dependent, reproducible and statistically significant increases (MN frequency increase reaching $p<0.05$ when compared to negative control values) in micronucleus frequencies were observed independent of the metabolic activation status for both the HHP and 1R6F.
- The HHP aerosol was 35.5-fold less genotoxic in the presence, and 36.3-fold less genotoxic in the absence, of a metabolic activation, compared to 1R6F Reference Cigarette smoke, based on the ECMN3, measured on a per puff basis.

4. CONCLUSIONS

- Chemical analysis revealed substantial reductions in the numbers and levels of selected toxicants within the HHP aerosol, with an average reduction of 97% for WHO TobReg9 analytes, and 6 out of 9 analytes falling below the limit of quantification (LOQ). These reductions translated into significant decreases in toxicological outcomes when compared to 1R6F smoke.
- The HHP aerosol exhibited significantly lower cytotoxicity, being 73 times less cytotoxic than 1R6F cigarette smoke on a per puff basis. As expected, 1R6F cigarette smoke was highly mutagenic and genotoxic, while the HHP aerosol showed significant reductions in genotoxicity and was not mutagenic in TA98 and TA100 under the test conditions.
- The data clearly demonstrate differences between 1R6F cigarette smoke and HHP aerosol emissions in terms of in vitro toxicity.
- These findings suggest that HHP could play a significant role in the tobacco harm reduction strategy.

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