Comparative Genotoxicity Assessment of Heated Tobacco Products (HTPs) and 1R6F Reference Cigarettes Using *In Vitro* Micronucleus Assay in TK6 and CHO Cell Lines.

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Background and purpose

This study investigates the genotoxic responses of two distinct cell lines, TK6 and CHO, to aerosols from heated tobacco products (HTPs) and smoke from the 1R6F reference cigarette. Using *in vitro* micronucleus testing as per OECD 487 guidelines, we compared the genotoxic effects of three commercial tobacco flavoured heat-not-burn (HTP) products—with the particulate phase (PP) of 1R6F smoke. The 1R6F data highlighted the limitations of traditional scoring methods, prompting the use of flow cytometry for more precise micronucleus detection. Our results demonstrated dose-dependent responses across different exposure schedules in both cell lines, with cytotoxicity levels found to be consistent between CHO and TK6 cells. These findings provide valuable insights into the differential genotoxic effects of aerosols from HTPs compared to conventional cigarette smoke, emphasizing the advantages of flow cytometry.

Materials and Methods

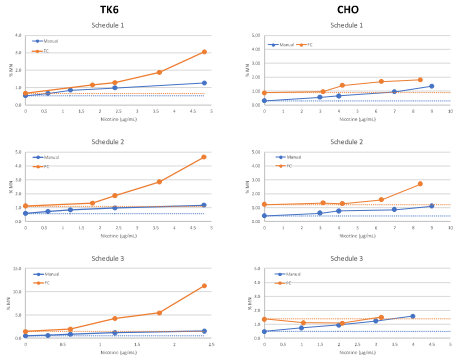
- **Test Products**: The study tested three commercial tobacco flavoured HTPs (HTP Product 1, HTP Product 2 & HTP Product 3). 1R6F reference cigarettes were used for the comparison between manual count and flow cytometric micronuclei assessment to validate the experimental approach-
- **Cell Lines**: Two cell lines, TK6 (human lymphoblastoid) and CHO (Chinese hamster ovary), were used for the assays.
- **Smoking Protocol**: The 1R6F cigarette smoke was generated using a rotary smoking machine, with the particulate phase (PP) extracted using DMSO (92 mm filter pads). The same method was applied to the aerosol collection [gas vapour phase (GVP) + PP] from the HTPs. The stock concentration after sample generation was 100 mg/mL for both PP and GVP, they were mixed in a 1:1 ratio. The top dose (1,000 µg/mL) was diluted to achieve a dose response. Cells were exposed to varying concentrations of these extracts.
- **Genotoxicity Testing**: The *in vitro* micronucleus test was performed following OECD 487 guidelines, with 3 distinct exposure schedules for each cell line. Flow cytometry was employed for micronucleus assessment.

Results

1. Manual scoring vs Flow cytometry

The automated MN assay exhibited a higher %MN compared to the manual method in TK6 cells. The dashed lines indicate the %MN of solvent controls for each method (Blue: Manual, Orange: Flow cytometry). The similar pattern was observed for %Cytotoxicity from both manual scoring and flow cytometry (data not shown). As for Schedule 3 in MN CHO cells, the data was not applicable for analysis due to a high percentage of cytotoxicity (OECD 487).

Figure 1. Manual vs Flow cytometry: 1R6F

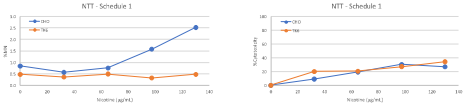


2. HTP Product 1

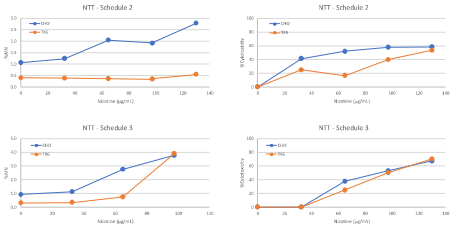
Dose-Response Observation: A dose-dependent increase in micronucleus formation was observed in CHO cells across all three schedules. In TK6 cells, a dose response was only noted in schedule 3.

Cytotoxicity: Cytotoxicity levels were consistent between CHO and TK6 cells.

Figure 2. Dose-response curves for HTP Product 1 in CHO and TK6 cells across three schedules.



Results

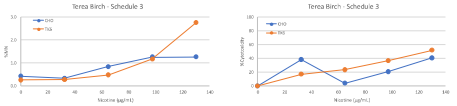


3. HTP Product 2

Dose-Response Observation: Dose-dependent responses were observed in both CHO and TK6 cells, but only in schedule 3.

Cytotoxicity: Cytotoxicity levels were similar between the two cell lines.

Figure 3. Comparison of dose-response curves for HTP Product 2 CHO and TK6 cells in schedule 3.

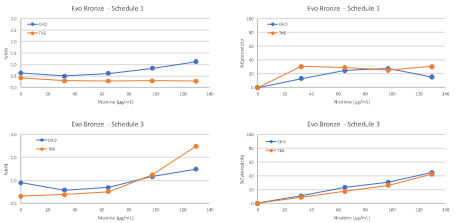


4. HTP Product 3

Dose-Response Observation: A dose response was noted in CHO cells in schedule 1 and 3. TK6 cells showed a dose response in schedule 3.

Cytotoxicity: Cytotoxicity levels were comparable between the CHO and TK6 cells.

Figure 4. Dose-response curves for HTP Product 3 in CHO and TK6 cells across schedules 1 and 3.



Conclusions

This study identified varying genotoxic responses among different HTPs and the 1R6F reference cigarette. The initial 1R6F data revealed the limitations of traditional micronucleus scoring methods, prompting the adoption of flow cytometry for more robust detection.

HTP Product 1 consistently demonstrated dose-dependent genotoxic effects, especially in CHO cells, whereas HTP Products 2 and 3 showed more selective responses. Despite differences in genotoxicity, cytotoxicity levels were similar across the CHO and TK6 cell lines, indicating a uniform cellular response to HTP aerosols.

These findings underscore the advantages of using flow cytometry for robust micronuclei detection. Genotoxicity was detected in both CHO and TK6 cell lines for all HTP products tested, despite variability in their responses. In conclusion, commercial HTP products were found to be genotoxic; however, they required higher concentrations than combustible cigarettes to induce similar genotoxic effects.

Reference

1. Bryce et al., 2007. "*In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity", Mutation Research. 630(1-2): 78–91.

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4. Smart et al., 2020. "Development of an integrated assay in human TK6 cells to permit comprehensive genotoxicity analysis *in vitro*", Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 849.503129.

5. OECD Guideline for the Testing of Chemicals No. 487, 2023. "*In Vitro* Mammalian Cell Micronucleus Test".

The previous title of the poster was "Comparative Analysis of Heated Tobacco Products and 1R6F Reference Cigarette Responses in TK6 and CHO cell lines utilizing High Throughput Flow cytometry and Manual Counting for the *in vitro* Micronucleus Assay".