RAPID *IN VITRO* TOXICOLOGICAL SCREENING USING TOXTRACKER TO DETERMINE THE EFFECT OF REPEATED FREEZE/THAW OF COMBUSTIBLE CIGARETTE EXTRACT

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Abstract

The freeze/thaw cycles of combustible cigarette extract (CCE) may lead to changes in the chemical composition through the formation of ice-water interfaces, phase separation, pH induced changes and chemical degradation. These changes may alter the outcome of *in vitro* toxicological assays. To assess the impact of repeated freeze and thaw on CCE at the DNA level using the stem cell-based reporter assay ToxTracker.

Combustible reference cigarettes (1R6F) (n=3) were smoked using the Health Canada standard regimen. All three sample collections [particulate phase (PP), gas-vapor phase (GVP) and the 1:1 combined phase (PP+GVP)] were applied to the ToxTracker assay. Samples were extracted and divided into two; half was applied to the ToxTracker assay within 1hr of sample generation and the other half underwent 3 freeze thaw cycles before applying to the ToxTracker assay. Cells were treated for 24hr (both +/- S9). Green-fluorescence protein (GFP)reporter gene induction and cytotoxicity was assessed using flow cytometry.

Fresh PP (-S9) samples induced the Srxn1 reporter (indicative of oxidative stress) 6-fold and the Ddit3 reporter (indicative of protein damage) 10-fold at 200µg CCE/mL; S9 decreased the Srxn1 response to 12-fold GFP-induction. Fresh PP+GVP (+S9), induced Srxn1 and Blvrb (indicative of oxidative stress) 10 and 4-fold, respectively at 200µg CCE/mL. Sample freeze has no effect on Srxn1 induction while Ddit3 increased GFP-induction from 6 to less than 2-fold (-S9). No significant changes between fresh (+S9) and freeze/thaw (+S9) for PP or PP+GVP samples was observed.

This data indicates that utilizing a "fresh" sample for *in vitro* toxicology testing will allow for the test system to be treated with a CCE that more closely resembles the chemical profile inhaled by cigarette smokers.

Introduction

Regulatory testing of combustible cigarettes requires both the particulate phase (PP) and the gas vapour phase (GVP) that are generated are used to assess the *in vitro* mutagenicity, genotoxicity and cytotoxicity of combustible tobacco products (1). Currently, there is no definitive proof that repeated freezing and thawing of the PP sample has any effect on the outcome of the *in vitro* assay.

Crooks *et al*. conducted a study in 2013 to assess the impact of freezing and thawing of the combustible PP and GVP samples in *in vitro* toxicological assays, however, the differences observed in the classical assays were not significant. In this study the aim was to determine if there is a difference in the mode-of-action between fresh and frozen samples that may not be apparent in the traditional *in vitro* toxicological assays.

Traditional *in vitro* toxicological assays do not detect changes in protein damage, DNA damage, oxidative stress or cellular stress to differentiate the effect of freeze/thaw on sample integrity. It is possible that the freeze/thaw process affects the sample to the point that cellular damage that cannot be detected by traditional *in vitro* assays but can be detected at the gene level by affecting specific cellular signaling pathways.

ToxTracker (Figure 1) is a stem cell-based reporter assay that provides mechanistic insight into the mode-of-action of genotoxic properties of chemicals (2,3), may be able to differentiate between freshly generated samples and those that have undergone repeated freeze thaw cycles. The assay utilizes 6 different reporter genes that are tagged with green fluorescent protein and when induced the degree of induction can be measured via flow cytometry.

Figure 1: Different Cell Signaling Pathways in the ToxTracker Assay

Results

The 1R6F aerosol was generated using a mainstream intense protocol and the PP, GVP and PP+GVP extracts were applied to the ToxTracker cells within 1 hr. The fresh PP induced the oxidative stress pathways (Srxn1 and Blvrb) 20-fold and 3-fold, respectively and the protein damage pathway (Ddit3) 6-fold at 200ug/mL compared to control, without S9. In the presence of S9, the induction of Srxn1 decreased 10-fold (Figure 2). There was no GFP-induction or decrease in cytotoxicity with GVP (Figure 3).

Figure 2: Fresh 1R6F PP, GRP-induction and Cytotoxicity (+/- S9)

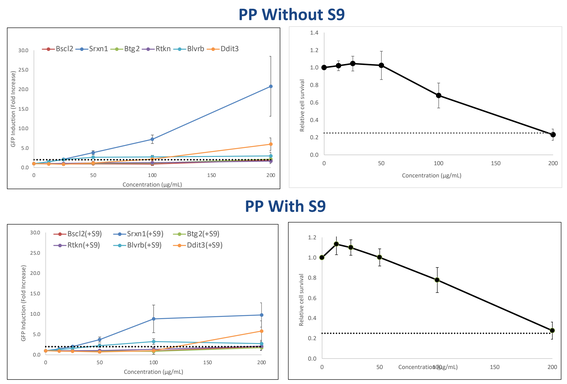
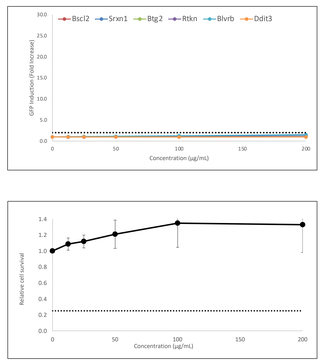
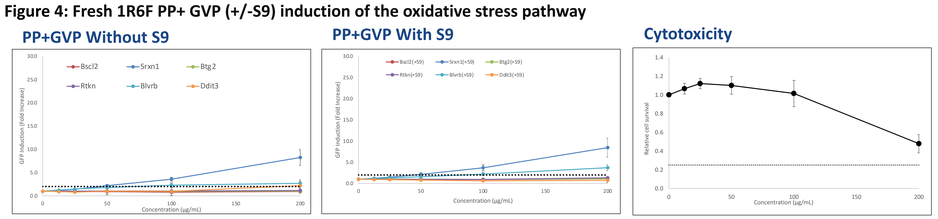


Figure 3: Fresh 1R6F GVP, GFP-induction and Cytotoxicity (-S9)



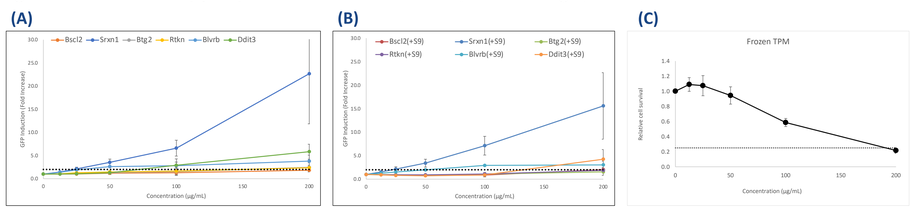
When the PP and GVP phases are combined in a 1:1 ratio the effect observed with PP only is decreased. The induction of the oxidative stress pathway is decreased to 10-fold with/without S9. Cytotoxicity decreased with increasing concentration (Figure 4).

Figure 4: Fresh 1R6F PP+ GVP (+/-S9) induction of the oxidative stress pathway



PP samples that underwent 3 freeze thaw cycles induced the oxidative stress pathway (Srxn1 and Blvrb) by 23-fold and 4-fold, respectively and the protein damage pathway 6-fold (Figure 5a). The degree of induction is not statistically different from fresh PP samples. The presence of S9 decreased the Srxn1 induction to 15-fold and Ddit3 to 4-fold (Figure 5b). A concentration dependent decrease in cytotoxicity was observed with/without S9 (Figure 5c).

Figure 5: Frozen 1R6F PP (+/- S9) induces oxidative stress and protein damage pathways



Methods

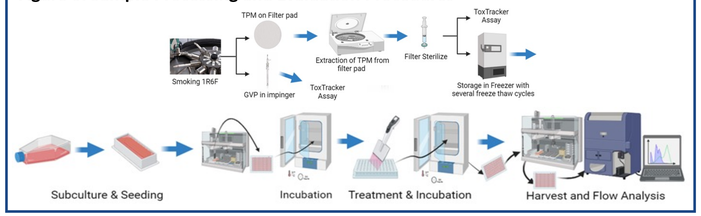
Kentucky reference cigarettes were smoked using the Health Canada intense smoking regime. The particulate phase (PP) was collected on 92mm Cambridge filter pads and the gas vapor phase (GVP) in 20mL PBS buffer. The PP phase was extracted with DMSO. The PP +GVP sample a 1:1 mixture of PP and GVP.

For the "fresh" experiments, the extract was applied to the test system within 1hr of collection, while for the "frozen" experiments the PP extract was frozen at -80°C for 1 week and then thawed to room temp. This cycle was repeated 3 times to mimic the sample shipping process.

For the ToxTracker assay, varying concentrations of the "fresh" PP, GVP or PP+GVP extract, or "frozen" PP were applied to each of the six reporter cell lines (+/- S9) in the ToxTracker assay. Following a 24hr incubation, reporter gene (GFP) induction and cytotoxicity were assessed by flow cytometry (Figure 6). Results were normalized to the wild type (no GFP tag) cell line to account for any autofluorescence in the sample and results displayed as an increase/decrease in (GFP)-induction and relative cell survival.

The maximum concentration of 1R6F extract (PP) applied to the test system was 200ug/mL.

Figure 6: Sample Processing and Evaluation Procedures



Conclusions

- **Effect of freezing 1R6F PP fraction:** No significant change in the oxidative stress pathway between fresh sample and samples that have undergone several freeze thaw cycles.
- **Effect of 1R6F on Protein Damage:** The fresh and frozen PP sample induced the protein damage pathway (Ddit3).
- **Effect of 1R6F on Cytotoxicity:** Cytotoxicity decreased in a concentration dependent manner with increasing concentrations (PP & PP+GVP).
- **Effect of Dilution:** Combining the PP and GVP in a 1:1 ratio results in a 50% decrease in the induction of the oxidative stress pathway.

References

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2. Hendriks, G., Atallah, M., Morolli, B., et al. (2012). The ToxTracker Assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences: 125(1):285-298.
3. David E. Smart, Stela Bozhilova, Fabio Miazzi, Linsey E. Haswell, Marianna D. Gaca, David Thorne, Damien Breheny, (2022). Application of ToxTracker for the toxicological assessment of tobacco and nicotine delivery products. Toxicology Letters, 358:59-68