Transcriptomic Effects of Menthol *vs.* Non-Menthol Cigarette Smoke on Human Alveolar Epithelial Cells

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Introduction

- Menthol makes up ~40% of cigarette sales in the U.S. (**Fig. 1**)
- **80% of Black American smokers** smoke menthol cigarettes
- **Study aim**: Investigate transcriptomic impact of menthol *vs.* non-menthol cigarette smoke exposure on human immortalized alveolar epithelial cells (hAECs)
- Cigarettes Used: commercial menthol and non-menthol cigarettes from American Spirit
- Initial Evaluation: Reverse transcription-quantitative PCR (RT-qPCR) to measure the mRNA levels of *CYP1A1* and *AHRR,* two genes that are known to be induced after smoke exposure, to confirm that we are using the proper concentrations of CSE
- Final Evaluation: Transcriptomic sequencing to compare the molecular effects of menthol *vs.* non-menthol smoke.

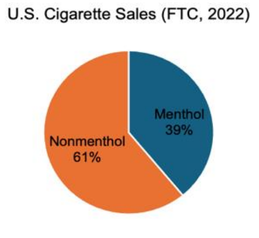
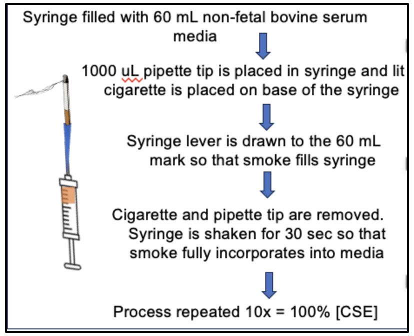


Fig. 1. Menthol smoking product sale frequency. Menthol cigarettes make up 40% of all total U.S. cigarette sales and over 50% of all e-cigarette sales.

Methods

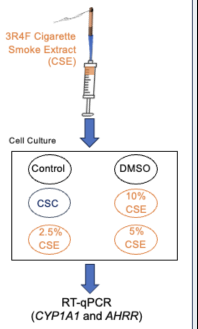


The Offringa lab previously used purchased cigarette smoke concentrate (**CSC**) , but it is not available for menthol cigarettes. There-fore, our first step is to see how cigarette smoke extract (**CSE**) made in the lab (method outlined in **Fig. 2**) compares to purchased CSC. For this comparison, we study the mRNA levels of *CYP1A1* and *AHRR*, two genes induced by tobacco smoke.

STEP 1: Comparison of CSE to CSC

Goals of Part 1:

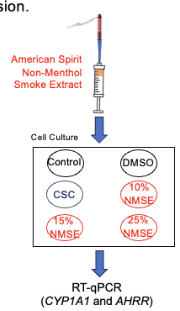
- CSC is a prepared solution (20 ug/mL) from the University of Kentucky commonly used in cigarette exposure studies
- CSC is not available in a menthol form. Therefore, we purchased commercial American Spirit menthol and non-menthol cigarettes for this study. As these are not available in CA, it took time to get these from New Mexico.
- In the meantime, we decided to compare different CSE concentrations made from cigarette 3R4F, used by University of Kentucky to find a general concentration range that yields an effect on AECs comparable to our usual 20 ug/ml CSC. We assessed effects by testing mRNA levels of *CYP1A1* and *AHRR.*



STEP 2: Comparison of American Spirit CSE Concentrations to CSC

Goals of Part 2:

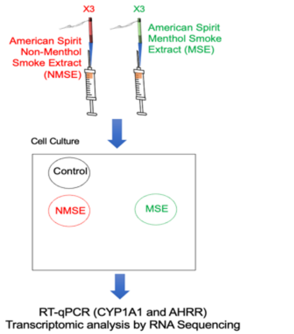
- Because there is no menthol version of 3R4F cigarettes, we chose organic American Spirit cigarettes for the menthol *vs.* non-menthol comparison. This avoids the potential confounding effect of pesticides.
- Using the 3R4F concentrations as a guide, we will determine concentration that most closely mimics effects of CSC on *CYP1A1* and *AHRR* expression.
- Compare triplicates of non-menthol CSE using 3 cigarettes from the same cigarette pack to determine replicability of CSE extraction process



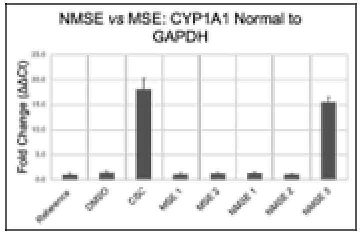
STEP 3: Comparison of Non-Menthol CSE *vs.* Menthol CSE

Goals of Part 3:

- Compare triplicates of menthol and non-menthol CSE using 3 cigarettes from the same cigarette pack and determine replicability of CSE extraction process and gene induction using *CYP1A1* and *AHRR* RT-qPCR.
- Use RNA Sequencing to determine potential differences in the effect of menthol *vs.* non-menthol smoke on hAECs



Results



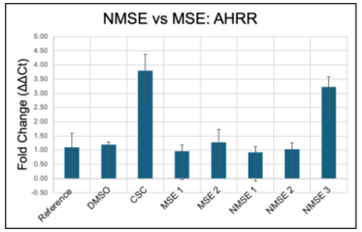


Figure 3 compares the gene expression of *CYP1A1* (top) and *AHRR* (bottom) across the exposures at 48 hours. American Spirit menthol smoke extracts (MSEs) from both cigarettes did not have a significant difference in the fold change compared to the control (reference) ($p>0.01$). However, one of the non-menthol smoke extracts from cigarette 3 (NMSE 3) had a significantly different fold change from reference and no significant difference compared to CSC ($p<0.01$).

Conclusions

In testing 10% American Spirit non-menthol vs menthol CSEs, most cigarettes showed no meaningful gene induction, but one non-menthol CSE produced results comparable to CSC.

Variability in CSE preparation could be due to differences in cigarette burn length (e.g., NMSE3) or inconsistencies in the manual shaking process during smoke media incorporation

Next Steps: Variability in CSE preparation could be due to differences in cigarette burn length (e.g., NMSE3) or inconsistencies in the manual shaking process during smoke media incorporation.

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