COMPARISON OF THE BACTERIAL MUTAGENICITY OF MAINSTREAM WHOLE SMOKE FROM CIGARETTES WITH DIFFERENT LEVELS OF MENTHOL

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ABSTRACT #1409

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Menthol is widely used in the pharmaceutical, cosmetic, food and tobacco industries and is generally regarded as safe (GRAS) for these applications. This study was conducted to compare the mutagenicity of mainstream whole-smoke (WS) and wet total particulate matter (WTPM) from two sets of experimental cigarettes to determine if menthol has an effect on this endpoint. The first pair of experimental cigarettes (delivering ~ 6 mg WTPM / cig) included a mentholated (0.6% w/w menthol) and a comparable non-mentholated control. The second set consisted of four experimental cigarettes (delivering ~ 9 mg WTPM / cig) with increasing levels of menthol (0.1% - 0.7% w/w) and a non-mentholated control. Within each set, the cigarettes were comparable in construction, composition, and WTPM deliveries with added menthol spanning typical user levels. All cigarettes were smoked under ISO puff profile (35 mL volume, 2 second duration and 1 minute interval) on a VITROCELL® VC10 smoking robot, with WTPM being pad collected and extracted in dimethylsulfoxide. TA98 and TA100 were exposed to WS or WTPM with metabolic activation (S9+) utilizing either the VITROCELL® Ames exposure modules and whole-smoke (WS) dilution system or the preincubation method (WTPM). The WS specific activity (revertants / µg) from the first pair of sample cigarettes showed the mentholated cigarette had significantly lower activity then the non-mentholated control (TA98, p = 0.0027; TA100, p = 0.0297). For the second set of cigarettes, only small differences in WS activities were observed, with a trend of decreasing activity with increasing menthol that was not statistically significant. No differences were detected for WTPM specific activities. No significant differences in WS activities occurred when cigarettes were removed from packs and conditioned (18 hours, 23°C, 60% RH) prior to smoking. With no changes in smoke delivery or cytotoxicity and with the reduction of menthol from the tobacco during conditioning, these results suggest menthol may have a role in the observed reduction of WS Ames activity.

INTRODUCTION

The potential effect menthol may have on the genotoxicity / mutagenicity of cigarette smoke has been studied previously in the Ames Assay; however, these studies typically utilized collected smoke condensates[1, 2]. Consistent results have revealed no differences in smoke mutagenic activity; however, collecting smoke particulates and separating them from the gas phase may change the chemical composition and any dynamic interactions that undoubtedly occur between the chemical entities present in both phases. Ideally, exposing the *Salmonella* tester strains directly to fresh whole cigarette smoke would be the preferred method to study any potential menthol effects. Systems and methods are available[3] for whole smoke delivery and exposure and were applied in this study.

MATERIALS & METHODS

CIGARETTE SMOKE PREPARATIONS & EXPOSURES:

All cigarettes were smoked on a VITROCELL® VC10 smoking robot following ISO puff profile: 35 mL puff volume, 2 second draw, 1 minute puff interval.

Cigarettes were either smoked immediately after removal from sealed packs ("Fresh") or removed from packs and allowed to condition at least 18 hours at 60% relative humidity (RH) and 23°C prior to smoking ("Conditioned").

Wet Total Particulate Matter (WTPM) collected on Cambridge filter pads, extracted in dimethylsulfoxide (DMSO) to a final concentration of 40 mg / mL, stored at -80°C prior to analysis.

Whole Smoke (WS) exposures performed on a VITROCELL® VC10 with WS dilution system and Ames Exposure Modules. Dilution air flows ranged from 0.5 L / min – 4.0 L / min.

Whole Smoke doses determined by:

1) WTPM delivery, dilution air flow, number of cigarettes and number of puffs per cigarette (calculation spreadsheet provided by VITROCELL®), or
2) Relationship between Photometer readings (µV) and fluorescence (Ex/Em: 355/485) of DMSO captured smoke constituents.

AMES ASSAY[3, 4]:

Post-mitochondrial supernatant, Aroclor 1254-induced male Sprague-Dawley rat liver in 0.15M KCl (Moltox; Boone, NC).

S9-Mix: 33mM KCl, 8mM $MgCl_2$, 5mM Glucose-6-phosphate, 4mM NADP, sodium phosphate buffer (0.1M, pH 7.4), S9 fraction @ 5% v/v (WTPM) or 30% v/v (WS, or as noted in figure legend).

Preincubation Ames assays (WTPM only): 100 µL of *Salmonella* strains TA98 or TA100, 500 µL S9-Mix (5% v/v), 25 µL WTPM or DMSO, 30 minute preincubation @ 37°C, 250 rpm shaking in capped tubes, followed with the addition of histidine / biotin top agar (2.5 mL) and plating onto minimal glucose agar plates.

WS Exposures: TA98 or TA100 @ ~$2X10^8$ - $2X10^9$ bacteria / mL in 100 µL S9-mix spread on 0.4% minimal glucose agar plates (35 mm) supplemented with 0.05 mM Histidine / Biotin, exposed to WS from three experimental cigarettes.

Revertant colonies counted (Artek 880 colony counter) after 48 hours of incubation @ 37°C.

Activity calculated from linear portion of the dose response curve and compared using GraphPad Prism v. 5.02 (slope analysis, two tailed; for comparisons, statistical significance @ $p < 0.05$).

VIABILITY ASSAY:

TA98 and TA100 were plated on nutrient agar plates at approximately 200 – 400 bacteria per plate, in the presence of S9.

Bacteria exposed to mainstream WS from three experimental cigarettes and viable colonies were counted after 48 hours of incubation @ 37°C.

RESULTS

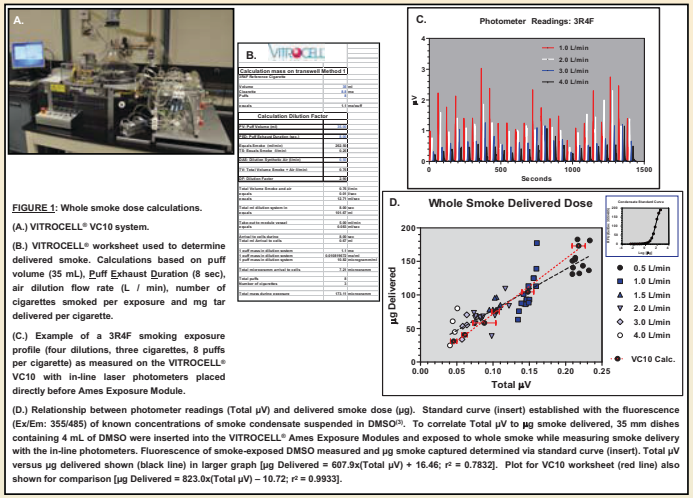

FIGURE 1: Whole smoke dose calculations.

(A.) VITROCELL® VC10 system.

(B.) VITROCELL® worksheet used to determine delivered smoke. Calculations based on puff volume (35 mL), Puff Exhaust Duration (8 sec), air dilution flow rate (L / min), number of cigarettes smoked per exposure and mg tar delivered per cigarette.

(C.) Example of a 3R4F smoking exposure profile (four dilutions, three cigarettes, 8 puffs per cigarette) as measured on the VITROCELL® VC10 with in-line laser photometers placed directly before Ames Exposure Module.

(D.) Relationship between photometer readings (Total µV) and delivered smoke dose (µg). Standard curve (insert) established with the fluorescence (Ex/Em: 355/485) of known concentrations of smoke condensate suspended in DMSO[3]. To correlate Total µV to µg smoke delivered, 35 mm dishes containing 4 mL of DMSO were inserted into the VITROCELL® Ames Exposure Modules and exposed to whole smoke while measuring smoke delivery with the in-line photometers. Fluorescence of smoke-exposed DMSO measured and µg smoke captured determined via standard curve (insert). Total µV versus µg delivered shown (black line) in larger graph [µg Delivered = 607.9x(Total µV) + 16.46; $r^2 = 0.7832$]. Plot for VC10 worksheet (red line) also shown for comparison [µg Delivered = 823.0x(Total µV) – 10.72; $r^2 = 0.9933$].

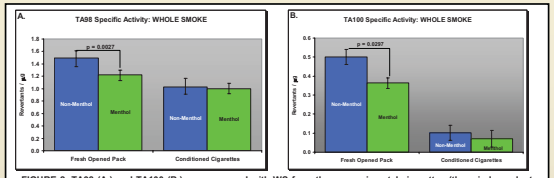

FIGURE 2: TA98 (A.) and TA100 (B.) were exposed with WS from three experimental cigarettes (three independent experiments). Differences between non-mentholated and mentholated cigarettes are apparent while no significant differences were seen for conditioned cigarettes. Lower activities in conditioned cigarettes, specifically TA100, are the result of exposures with different bacteria cultures and S9 preparations and not an effect of conditioning. Further analysis of the TA100 results led to the decreasing of the S9-fraction from 30% down to 5% in final S9-mix (v/v) in future exposures.

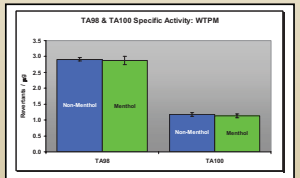

FIGURE 3: Pad collected WTPM from "fresh" cigarettes was extracted in DMSO. *Salmonella* strains TA98 and TA100 were exposed to WTPM using the pre-incubation method in the presence of S9 (5%). Results from three independent assays show no differences in specific activity (TA98, p = 0.8335; TA100, p= 0.7889).

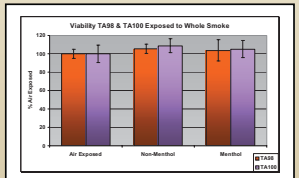

FIGURE 4: TA98 and TA100 were plated on nutrient agar plates and exposed to WS from three experimental cigarettes taken from freshly opened packs. No differences in cell viability were observed when compared to air exposed controls (TA98, p = 0.4289; TA100, p = 0.5856).

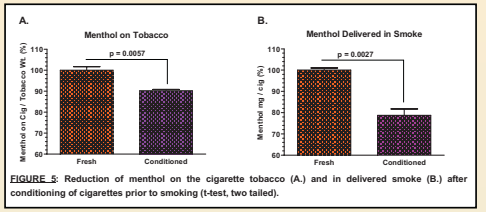

FIGURE 5: Reduction of menthol on the cigarette tobacco (A.) and in delivered smoke (B.) after conditioning of cigarettes prior to smoking (t-test, two tailed).

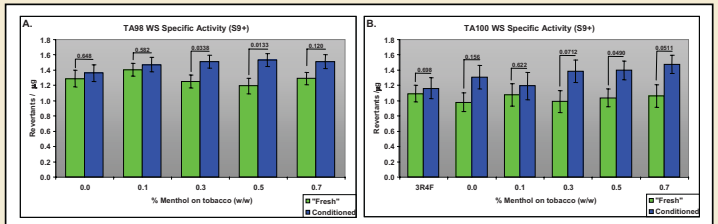

FIGURE 6: Exposures with second series of experimental cigarettes with increasing levels of menthol (% menthol on tobacco, w/w; 0 – 0.7%). TA98 (A.) and TA100 (B.) were exposed with WS from three cigarettes (TA98 three trials, TA100 two trials). TA98 exposures utilized 30% (v/v) S9-mix and air dilution flow rates of 0.5, 1.0, 1.5 and 2.0 L / min. TA100 exposure parameters were changed from previous exposures due to observed toxicity of S9 (30%) to the *Salmonella*. TA100 exposures utilized 5% (v/v) S9-mix and air dilution flow rates of 1.0, 2.0, 3.0 and 4.0 L / min. No significant differences in WS activity were observed when samples were "fresh" (TA98, p = 0.726; TA100, p = 0.983) or "conditioned" (TA98, p = 0.718; TA100, p = 0.612). Statistically significant differences were seen when comparing respective cigarettes smoked "fresh" versus "conditioned" ($p < 0.05$, t-test, two tailed).

SUMMARY

- Differences in Whole Smoke (WS) specific activities (S9+), measured in *Salmonella* strains TA98 and TA100, were observed between mentholated and non-mentholated cigarettes, as well as "fresh" and "conditioned" mentholated cigarettes.
- Statistically significantly lower WS activity was observed for a mentholated cigarette when compared to its non-mentholated control (Figure 2). This difference was not observed for cigarettes that were allowed to condition (23°C, 60% RH, ≥18 Hours) prior to smoking or from WTPM collected from "fresh" cigarettes (Figures 2 & 3).
- Menthol is known to possess antimicrobial activity[5]. The differences between mentholated and non-mentholated ("fresh") WS activity were not the result of smoke toxicity since cell viability for the mentholated and non-mentholated WS exposed *Salmonella* cigarette were the same (Figure 4).
- Menthol levels were reduced ~ 10% on the cigarette tobacco and ~ 21% in delivered smoke after cigarette conditioning (Figure 5), suggesting menthol may have a role in the observed differences of WS Ames activity through some yet to be determined mechanism(s) or pathway(s).
- Second set of experimental cigarettes did show statistically significant differences between "fresh" and "conditioned" WS activity; however, there were no statistical differences in WS activity between "fresh" mentholated and non-mentholated control (Figure 6), as seen earlier (Figure 2). This could be the result of a different tobacco blend and tar deliveries (~ 6 mg vs. ~ 9 mg) when compared to the initial pair of test cigarettes (Figures 2 – 5).

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