COMPARISON OF THE BACTERIAL MUTAGENICITY OF WHOLE SMOKE, GAS-VAPOR PHASE AND SMOKE CONDENSATES FROM A MENTHOLATED AND NON-MENTHOLATED CIGARETTE

Robert D. Leverette

A.W. Spears Research Center, Lorillard Tobacco Company, Greensboro, NC 27405, USA

ABSTRACT

Menthol is widely used in the pharmaceutical, cosmetic, food and tobacco industries and is generally regarded as safe (GRAS) for these applications. Menthol does possess some analgesic properties as well as other receptor-mediated biological effects, which are responsible for its characteristic "cooling" sensation when applied to the skin, ingested or inhaled. However, it is unclear if menthol, as delivered from a typical mentholated cigarette has any effect on smoke-induced biological endpoints. This study was conducted to compare the reverse bacterial mutagenicity (Ames Assay) of collected wet total particulate matter (WTPM), whole smoke (WS) and gas-vapor phase (GVP) from a non-mentholated and mentholated cigarette. The cigarettes used in this study were comparable in construction, composition, WTPM deliveries (approximately 6.4 mg WTPM), and differed only in the presence (approximately 0.6% w/w) or absence of menthol. All cigarettes were smoked under the ISO puff profile (35mL puff volume, 2 second puff duration and a 1 minute puff interval) on a VITROCELL® VC10 smoking robot. WTPM from each cigarette type was collected on a Cambridge pad and extracted in dimethylsulfoxide. *Salmonella* strains TA98 and TA100 were exposed to serial dilutions of WTPM with metabolic activation (S9+). Whole smoke (TA98 and TA100, S9+) and GVP (TA100, S9-) exposures were performed on 35mm minimal glucose agar plates utilizing the VITROCELL® Ames exposure modules and smoke dilution system. There were no differences in the specific activities (revertants / µg) of the WTPM (TA98, p = 0.8335; TA100, p = 0.7889) or the GVP (p = 0.4595). Differences were seen in the whole smoke activities in both strains, with the mentholated sample having significantly lower specific activity than the non-mentholated cigarette (TA98, p = 0.0027; TA100, p = 0.0297).

INTRODUCTION

The potential effect menthol may have on the mutagenicity of cigarette smoke has been studied previously in the Ames Assay utilizing collected smoke condensates (1, 2). No differences in mutagenic activity were observed; however, collecting smoke particulates and separating them from the smoke 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 24°C prior to smoking ("Conditioned").
- Wet Total Particulate Matter (WTPM): Collected on Cambridge filter pads and extracted in dimethylsulfoxide (DMSO) at a final concentration of 40 mg / mL, aliquoted and stored frozen 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 @ 0.5, 1.0, 1.5 and 2.0 L / minute. Doses calculated based on WTPM delivery, dilution air flow, number of cigarettes and number of puffs per cigarette (calculation spreadsheet provided by VITROCELL®).
- Gas Vapor Phase (GVP) exposures identical to WS, but a Cambridge pad was placed inline prior to the smoke dilution system.

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, GVP).
- 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 (control), 30 minute preincubation @ 37°C, 250 rpm shaking in closed tubes, followed with the addition of histidine / biotin top agar (2.5 mL) and plating onto minimal glucose agar plates.
- WS and GVP Exposures: TA98 or TA100 @ ~2X10^8 bacteria / mL in 100 µL S9-mix (WS) or PBS (GVP, S9-) spread on 0.4% minimal glucose agar plates (35 mm) supplemented with 0.05 mM Histidine / Biotin. Bacteria were exposed to WS or GVP 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).

VIABILITY ASSAY:

- Performed identically as the WS exposures with the following differences: TA98 and TA100 were plated on nutrient agar plates at approximately 200 – 400 bacteria per plate, in the presence of S9.
- Bacteria were exposed to the mainstream WS from three experimental cigarettes and viable colonies were counted after 48 hours of incubation @ 37°C.

MENTHOL ANALYSIS:

- Menthol extracted from tobacco or collected WTPM with 0.1% anethole / methanol.
- Extract analyzed by GC - FID.

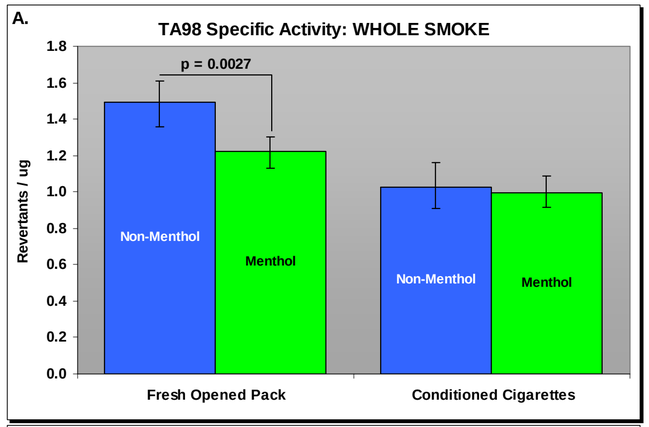

Figure 1: TA98 (A) and TA100 (B) were exposed to WS from three experimental cigarettes. Results are compiled from three independent experiments. Differences between non-mentholated and mentholated cigarettes are apparent in TA98 (p = 0.0027) and TA100 (p = 0.0297), fresh opened pack only. No significant differences seen for conditioned cigarettes (TA98, p = 0.7092; TA100, p = 0.5456). Lower activity in conditioned cigarettes, specifically TA100, is the result of exposures with different bacteria cultures and S9 preparations and not an effect of conditioning.

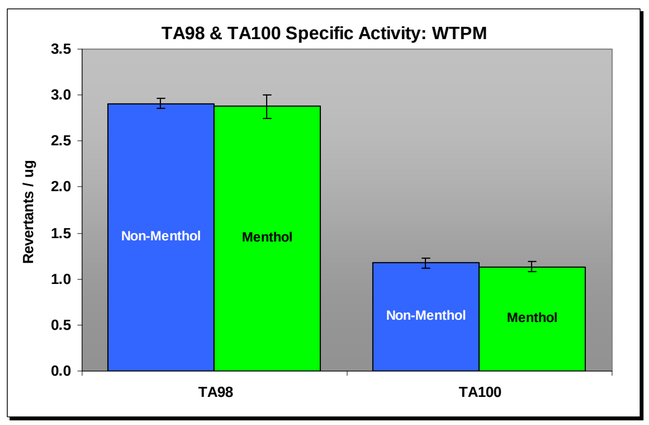

Figure 2: WTPM was collected from cigarettes taken from freshly opened packs. No differences in specific activity were detected (TA98, p = 0.8335; TA100, p= 0.7889).

RESULTS

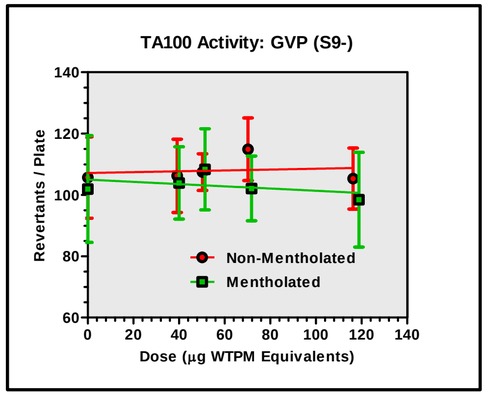

Figure 3: TA100 (S9-) was exposed to the GVP from three experimental cigarettes taken from freshly opened packs. Results are compiled from three independent experiments. No GVP activity was observed, as indicated by the regression lines, with no overall difference (p = 0.4595). However, it may be necessary to increase number of cigarettes for GVP exposures in order to detect any potential response.

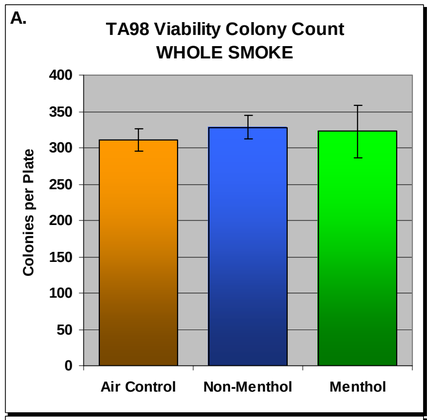

Figure 4: In order to determine if the differences observed between the WS activities of the non-mentholated and mentholated cigarettes were due to cell viability, TA98 (A) and TA100 (B) plated on nutrient agar plates were exposed to WS from three experimental cigarettes taken from freshly opened packs. No differences in cell viability were observed (TA98, p = 0.4289; TA100, p = 0.5856).

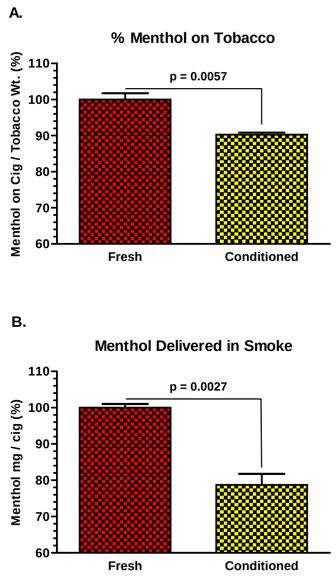

Figure 5: Reduction of menthol on the cigarette tobacco (A) and in delivered smoke (B) after conditioning of cigarettes prior to smoking (t-test, two tailed).

CONCLUSIONS

- WS specific activities (S9+), measured in *Salmonella* strains TA98 and TA100, were statistically significantly lower for the mentholated versus the non-mentholated cigarettes, freshly opened only (figure 1).
- The differences in WS activity for "Fresh" cigarettes were reduced if the experimental cigarettes were conditioned prior to smoking (figure 1).
- No differences in activity were observed for the WTPM collected from "Fresh" (unconditioned) experimental cigarettes (figure 2), as previously observed (3, 4).
- No differences were seen for GVP (TA100, S9-); however, it appears the number of cigarettes per exposure may need to be increased in order to detect any potential response (figure 3). We conclude that the vast majority of WS activity measured from three cigarettes is the result of the particulate matter, which was removed by the in-line filter used to generate GVP.
- The differences in WS activity are not the result of smoke toxicity since no differences in cell viability between the WS from the mentholated and non-mentholated cigarette were observed (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 reduction of WS Ames activity through some yet to be determined mechanism or pathway.

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