Effect of menthol on the penetration of tobacco carcinogens and nicotine across porcine oral mucosa ex vivo

Christopher A. Squier, Ph.D., D.Sc., Mary J. Mantz, M.S., & Philip W. Wertz, Ph.D.

Dows Institute for Dental Research, College of Dentistry, University of Iowa, Iowa City, IA

Corresponding Author: Christopher A. Squier, Ph.D., D.Sc., Dows Institute for Dental Research, College of Dentistry, University of Iowa, N406 DSB, Iowa City, IA 52242, USA. Telephone: 319-335-7388; Fax: 319-335-8895; E-mail: christopher-squier@uiowa.edu

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Abstract

Introduction: Menthol is a flavored tobacco additive claimed to mask the bitter taste and reduce the harshness of cigarette smoke. (Azzi, C., Zhang, J., Purdon, C. H., Chapman, J. M., Nitcheva, D., Hebert, J. R., et al., 2006, Permeation and reservoir function of 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) across porcine esophageal tissue in the presence of ethanol and menthol. Carcinogenesis, 27, 137–145). have shown that menthol increased the flux of tobacco carcinogens (TC) across porcine esophagus. As oral mucosa is exposed to both smoke and smokeless tobacco in tobacco users, the objective of this study was to determine whether menthol influenced the penetration of the TC nitrosornicotine (NNN) across porcine buccal (BM) and floor of mouth (FM) mucosa.

Methods: Porcine BM and FM were collected at slaughter, mounted in perfusion chambers (n = 7/group), and exposed to tritiated NNN (H-NNN; Amersham, activity 1 μCi/ml) and tritiated nicotine (H-nicotine; Sigma) in 3% nicotine/phosphate-buffered saline (0.01 M, pH 7.4) containing 0.01% unlabeled NNN (National Cancer Institute Chemical Carcinogen Repository) ± 0.08% menthol for 0.5, 1, 2, or 12 hr. K0 values (cm/min) were determined and statistically analyzed (analysis of variance, Tukey’s, p < .05).

Results: FM and BM permeability to both H-NNN and H-nicotine was significantly increased (p < .05) with addition of menthol over that of nicotine alone regardless of exposure times. Even short 30-min menthol exposure significantly increased the flux of both compounds, and this was maintained throughout the experiment.


Introduction

Menthol is a monocyclic terpene alcohol that occurs naturally in the plants of the Mentha species and is the major component of peppermint oil. It is used in products such as chewing gum, lozenges, and liniments because of the minty flavor, aroma, and cooling sensation that are produced when menthol contacts the skin and mucous membranes. As a tobacco additive, it has the effect of masking the bitter taste and reducing the harshness of cigarette smoke. It was first introduced into cigarettes in 1926 and claimed to make the smoke less irritating and soothing for sore throats (Wood, 1959). The popularity of mentholated cigarettes increased markedly in the 1950s with the introduction of brands, such as Kool and Salem, particularly among Blacks and women (Maxwell Associates, 1977). That popularity continues today; about 26% of all cigarettes sold are mentholated with disproportionate use by Black men (65.8%) and Black women (73.3%) and, to a lesser extent, by adult White women (27.4%) as compared with adult White men (17.7%; Giovino et al., 2004).

Earlier this year, federal legislation was passed giving the U.S. Food and Drug Administration (FDA) new powers to regulate the tobacco industry. This law prohibits the use of flavoring additives such as chocolate, cherry, and cloves in tobacco products but exempts menthol from the list of regulated flavoring additives. However, under the law, the FDA will be required to study the effects of mentholated tobacco products within 18 months (Bigg, 2009). Studies such as the present one are important to the understanding of the physiological effects of menthol on mucosal tissue.

Studies carried out over the past 15 years have shown that menthol is able to increase the transdermal (Kunta, Goskonda, Brotherton, Khan, & Reddy, 1997; Song, Gwak, & Chun, 2009; Yamato et al. 2009) and transbuccal (Shojaei, Khan, Lim, & Khosravan, 1999) penetration of dideoxycytidine, propofol, propanolol, and ofloxacin. More recently, Azzi et al. (2006) have shown that menthol increases the flux of the tobacco-related
nitrosamine, nitrosornornicotine (NNN), across porcine esophageal tissues that are directly impacted by cigarette smoke and where the harsh flavor and improving the taste of tobacco could contribute to increased consumption of cigarettes. Mentholated cigarettes also have higher tar and nicotine levels, and menthol smokers have higher cotinine levels and score higher on a measure of nicotine dependence than nonmentholated cigarette smokers (Ahijevych & Garrett, 2004; Ahijevych & Parsley, 1999). Additionally, Blacks who prefer mentholated cigarettes have higher cotinine levels (Ahijevych & Garrett) and exhibit a lower quit rate than White smokers who prefer non–mentholated cigarettes (Centers for Disease Control, 2002). Thus, a reduction in harshness and an improvement in taste of the smoke could encourage deeper inhalation and longer retention of cigarette smoke in the lungs that, in turn, could increase tar exposure and the amount of tobacco carcinogens (TCs) entering the blood stream. However, if menthol also acts as a permeabilizer for TCs, there would be increased exposure of the oral mucosa—tissues that are directly impacted by cigarette smoke and where oral cancer develops. Furthermore, if menthol also influences the absorption of nicotine, addiction may be increased, which would influence the consumption of cigarettes. Impact, the characteristic sensory effect or "bite" associated with smoking is a key component of the perception of strength of a cigarette (Wayne & Connolly, 2004). Industry studies have shown that menthol is capable of increasing nicotine impact in cigarette smokers (Philip Morris, 1995). This same study also showed that menthol increases impact on smoking in the absence of nicotine, suggesting that menthol alone could be addictive.

The objective of this study was to determine whether menthol might influence the penetration of the TC NNN and nicotine across nonkeratinized oral mucosa, the most frequent site for the development of tobacco-associated oral carcinoma (Mashberg, 1980; Squier, 1986).

## Materials and Methods

The menthol content of cigarettes marketed as “menthol cigarettes” ranges from 0.1%–1.0% (expressed as percentage of dry tobacco weight; Giovino et al., 2004). Menthol is only slightly soluble in water (≤0.1%), and although the solubility can be increased with glycerol or ethanol, we chose to incorporate it at a concentration of 0.08%, a level that approximates the lower range found in mentholated cigarettes. This also represents a concentration that remains in solution without cosolvents, thus avoiding the introduction of compounds that themselves might act as permeability enhancers or influence the penetration of the test compounds.

As nicotine is present in all tobacco products, solutions for all treatment conditions were prepared with 3% nicotine in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). This amount of nicotine was chosen as the average concentration reported in a range of tobacco products, actual values varying between 1% and 8% (percentage of dry tobacco weight; Gritz, Baer-Weiss, Benowitz, Van Yunatis, & Jarvi, 1981; Hecht, Ornaf, & Hoffman, 1974; Squier, 1986).

To study the effect of menthol on penetration of NNN, 3H-NNN (GE Healthcare, Piscataway, NJ), was added so as to achieve an activity of 1 μCi/ml and the solution doped with unlabeled NNN (National Cancer Institute Chemical Carcinogen Repository, Kansas City, MO) to produce a total concentration of 0.01%, which represents a level of NNN approximating that found in tobacco products (Hilfrich, Hecht, & Hoffman, 1977). Menthol (Sigma, St. Louis, MO) was added at a concentration of 0.08% to all solutions except for the controls, which were prepared as just described but without menthol.

To study the effect of menthol on penetration of nicotine, 3H-nicotine (Sigma) was added to the 3% nicotine PBS solution so as to achieve an activity of 1 μCi/ml, and menthol (Sigma) was added at a concentration of 0.08%. Controls were again prepared without menthol.

Porcine tissue, including esophagus, oral and vaginal mucosa, and skin, is a well-established model for the corresponding human tissues in terms of histology, ultrastructure, barrier function, and permeability (Perry et al., 2005; Squier, Manzt, Schlievert, & Davis, 2008). Porcine floor of mouth (FM) and buccal mucosa were collected at slaughter and utilized within 3 hr of harvest. Tissue disks in replicates of seven for each tissue/condition were mounted in continuous flow temperature-controlled perfusion chambers developed for mucosal permeability studies (Squier, Kremer, & Wertz, 1997; PermeGear, Inc., Hellertown, PA). They were maintained at 37 °C. One milliliter of the radiolabeled solution was applied to the epithelial surface of the tissue in the donor compartment and PBS (0.01 M, pH 7.4) pumped through the receptor compartment of the diffusion cell to collect any radiolabeled compounds permeating the tissue. This perfusate was collected at 1-hr intervals for up to 12 hr, and the penetration of the radiolabeled compound was determined by counting in a scintillation counter. The experimental design is summarized in Table 1.
The effect of menthol on the permeability of FM and buccal mucosa to nicotine and NNN was first determined by exposing the tissue to $^3$H-NNN and $^3$H-nicotine with 0.08% menthol and without menthol (controls) for a 12-hr period, during which time, a steady state is achieved. However, as mucosal exposure to tobacco components is of limited duration during normal tobacco use, other specimens were exposed to $^3$H-NNN menthol and $^3$H-nicotine menthol solutions for 0.5, 1, or 2 hr, after which time, the solutions were removed and replaced with $^3$H-NNN and $^3$H-nicotine without menthol for up to 6-hr total so as to determine the effect of menthol on penetration of NNN and nicotine after short- and long-term exposure.

Flux ($J$) was calculated at each sampling point from the relationship: $J = Q/A \cdot t$, where $Q$ is the quantity of compound traversing the tissue (dpm) in time $t$ (min) and $A$ is the area of exposed tissue in cm$^2$. The flux values were compared using an analysis of variance (ANOVA) and Tukey’s post test to determine which consecutive values were not significantly different, indicating that a steady state had been reached.

Table 1. Experimental design

<table>
<thead>
<tr>
<th>Treatment/tissue</th>
<th>Solution applied to epithelial surface</th>
<th>Exposure time</th>
<th>Solution removed</th>
<th>Replacement solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor of mouth or</td>
<td>$^3$H-nicotine + 3% unlabeled nicotine in 0.01 M PBS (no menthol)</td>
<td>12 hr</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor of mouth or</td>
<td>$^3$H-NNN + 0.01% unlabelled NNN + 3% unlabeled nicotine in 0.01 M PBS (no menthol)</td>
<td>12 hr</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor of mouth or</td>
<td>$^3$H-nicotine + 3% unlabeled nicotine in 0.01 M PBS + 0.08% menthol</td>
<td>30 min, 1, and 2 hr after application</td>
<td>Yes</td>
<td>$^3$H-nicotine + 3% unlabeled nicotine in 0.01 M PBS (no menthol)</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor of mouth or</td>
<td>$^3$H-NNN + 0.01% unlabelled NNN + 3% unlabeled nicotine in 0.01 M PBS + 0.08% menthol</td>
<td>30 min, 1, and 2 hr after application</td>
<td>Yes</td>
<td>$^3$H-NNN + 0.01% unlabelled NNN + 3% unlabeled nicotine in 0.01 M PBS (no menthol)</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. NNN = nitrosonornicotine; PBS = phosphate-buffered saline.

The flux values for the permeation of $^3$H-NNN and $^3$H-nicotine over the 12-hr sampling period for FM and buccal mucosa are shown in Figure 1. Steady state flux was achieved by Hour 2 and maintained throughout the sampling period for all treatments. The presence of menthol significantly increased ($p < .05$) the flux of both $^3$H-NNN and $^3$H-nicotine over that of controls with no menthol for both tissue types.

When menthol was removed after shorter exposure periods of 30 min, 1, and 2 hr, a steady state flux was reached by Hour 2 and...
Table 2. $K_r$ values* for floor of mouth (FM) and buccal (BM) mucosa to $^{3}$H-NNN and $^{3}$H-nicotine treated with 0.08% menthol for 30 min, 1, and 2 hr and a nonmentholated solution as a control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FM/$^{3}$H-NNN</th>
<th>BM/$^{3}$H-NNN</th>
<th>FM/$^{3}$H-nicotine</th>
<th>BM/$^{3}$H-nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>No menthol (control)</td>
<td>5.71 (±0.11; n = 12)</td>
<td>4.58 (±0.18; n = 10)</td>
<td>7.91 (±0.25; n = 10)</td>
<td>9.68 (±0.08; n = 5)</td>
</tr>
<tr>
<td>Menthol 30 min</td>
<td>6.79 (±0.24; n = 9)</td>
<td>5.91 (±0.29; n = 7)</td>
<td>8.41 (±0.36; n = 8)</td>
<td>11.1 (±0.16; n = 6)</td>
</tr>
<tr>
<td>Menthol 1 hr</td>
<td>6.92 (±0.43; n = 6)</td>
<td>7.25 (±0.45; n = 5)</td>
<td>9.42 (±0.16; n = 8)</td>
<td>11.2 (±0.12; n = 5)</td>
</tr>
<tr>
<td>Menthol 2 hr</td>
<td>7.94 (±0.27; n = 10)</td>
<td>7.20 (±0.46; n = 9)</td>
<td>9.64 (±0.27; n = 9)</td>
<td>10.9 (±0.15; n = 5)</td>
</tr>
</tbody>
</table>

Note. NNN = nitrosornicotine.
*Values are $K_r$ (±SD) × 10^{-4} (cm/min).
All $K_r$ values within a group significantly greater than the control for that group ($p \leq .001$).

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maintained throughout the 6-hr experiment for both tissue types and for both menthol/nonmenthol conditions (see Figure 2).

The permeability constants determined for the exposures described above are shown in Table 2. Permeability of both tissue types to $^{3}$H-NNN and $^{3}$H-nicotine in the presence of menthol for all exposure periods was significantly increased ($p \leq .001$) over that of nonmentholated controls.

Discussion

This study examined two consequences of using mentholated tobacco products, increased oral exposure to carcinogens, and increased nicotine delivery (and hence the potential for increased addiction). Recent studies have shown that menthol is able to increase the transdermal and transbuccal penetration of several drugs and that menthol increases the flux of the tobacco-related nitrosamine, NNN, across porcine esophagus. Menthol enhances transdermal absorption by affecting intracellular lipids or proteins and increasing the partitioning of drugs into the stratum corneum (Ahijevych & Garrett, 2004; Kaplun-Frischhoff & Toutou, 1997). Mechanism by which menthol increases transbuccal absorption is not clear but may enhance the partitioning of drugs across the transcellular pathway (Ahijevych & Garrett; Shojaei et al., 1999).

There is very little information in the literature on the effects of menthol on the uptake of carcinogens and nicotine in the oral tissues. We have described an increase in the uptake of both nicotine and NNN with addition of menthol, even after brief exposure. This may reflect short-term loading of a superficial epithelial reservoir that is facilitated in the presence of menthol and which then enhances flux for several hours after a relatively short surface exposure to tobacco. We have previously demonstrated that such a reservoir exists in the superficial layers of the oral epithelium (Squier, Kremer, Bruskin, Rose, & Haley, 1999). After rapid uptake of the nicotine and NNN into the surface layers of the epithelium, this “loading” is sufficient to maintain a flux of these compounds for several hours after removal of the menthol from the donor solution. Additionally, Scmeltz and Schlotzhauer (1968) showed that pyrolysis of menthol at high temperatures (860 °C, the burning temperature of tobacco) resulted in formation of carcinogenic polycyclic aromatic hydrocarbons (PAHs), such as pyrene, benzo[a]pyrene, and benz[a]anthracene. These PAHs, which have been shown to penetrate human buccal mucosa (van der Bijl & van Eyk, 1999), could contribute to the total quantity of carcinogens to which the mucosa is exposed.

Recent federal legislation prohibits addition of flavoring additives to tobacco products but exempts menthol from this ban while calling for the FDA to study medical effects and marketing of menthol and report their findings within 18 months (Bigg, 2009). Studies such as ours indicate that in the presence of menthol, there is an increase in the uptake of carcinogens and nicotine that have the potential to increase the risk of oral cancer and addiction. The claimed benefits of menthol in reducing the harshness of smoke and enhancing the taste must be weighed against the risks of increased exposure to carcinogens and nicotine.

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Declaration of Interests

None declared.

References


