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Original investigation

Impact of Menthol on Oral Nicotine Consumption in Female and Male Sprague Dawley Rats

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Abstract

Introduction: One of the preferable flavors in oral nicotine delivery systems is menthol which masks the harshness of tobacco. However, possible interactions between oral menthol and nicotine on intake and preference remain unclear. Therefore, we aimed to determine the impact of menthol on oral nicotine consumption.

Methods: Adult Sprague Dawley female and male rats (n = 8 per group) were given a choice of water or drug solution by using two-bottle free choice paradigm for 2 weeks: vehicle (5% ethanol), nicotine (20 mg/L), menthol (1 g/L) and mentholated nicotine groups. At the end of the study, plasma nicotine levels were determined.

Results: When rats were given a choice of nicotine or water, nicotine intake was similar between female and male rats. Menthol addition to nicotine solution significantly increased nicotine intake and preference in male but not female rats without a considerable effect on total fluid intake and body weight change in either sex. The average nicotine intake in male rats was 0.5 ± 0.05 and 1.4 ± 0.12 mg/kg/day for nicotine and menthol-nicotine combination ($p < .05$), respectively. The average nicotine intake in female rats was 0.6 ± 0.05 and 0.6 ± 0.03 mg/kg/day for nicotine and menthol-nicotine combination ($p > .05$), respectively. Plasma nicotine levels were not significantly different between the groups in either male (nicotine group: 20.8 ± 4.9, mentholated nicotine group: 31.9 ± 3.2 ng/mL) or female (nicotine group: 24.0 ± 3.3, mentholated nicotine group: 17.8 ± 2.9 ng/mL) rats ($p > .05$).

Conclusions: Menthol increases oral nicotine consumption in male, but not female, rats.

Implications: This study may provide data on the co-use of menthol and nicotine in smokeless tobacco, particularly oral dissolvable tobacco products.

Introduction

Nicotine addiction continues to be a major health problem causing an estimated annual 435 000 premature deaths in the United States and 5 million deaths worldwide.[1] Nicotine is the main active ingredient in tobacco smoke that leads to and maintains tobacco addiction.[2] Although the rate of smoking has decreased in the last 10 years, the smoke-free laws in the United States has encouraged the smokeless tobacco market. Indeed, a new generation of smokeless tobacco products has entered the US market, and grown in popularity, over the past several years.

The use of recent oral nicotine delivery systems such as snus products and dissolvable tobacco products is on the rise.[2–4] Dissolvable tobacco products are finely ground tobacco compressed into sticks, strips, and tablets (orbs) that dissolve in the mouth and do not require spitting.[5–7] These products deliver nicotine between 0.5 and 6 mg as they dissolve or melt in the consumer's mouth.[4,5,8,9] Dissolvable tobacco products usually contain various flavors (eg, menthol) and sweeteners to mask the harshness of tobacco and nicotine. There is an increasing interest in trying these flavored products.[3]

One of the preferable flavors in oral nicotine products is menthol due to its minty taste, cooling sensation and anesthetizing properties. Menthol, a monocyclic terpene alcohol, is found naturally in peppermint and corn mint plant oils. The typical menthol cigarette contains 2.9–19.6 mg of menthol,[10] and ~1 (0.03–2.3) mg of nicotine.[11] Smokeless pouched products have a menthol content of 1.6–5.1 mg/pouch and a nicotine content of 2–10 mg/pouch.[12] Another source of oral nicotine is nicotine replacement therapy products: gums and lozenges. Menthol flavored gum contains menthol (30 mg) and nicotine (2–4 mg) from the chewing gum.[13] While oral nicotine and menthol are subjected to first-pass metabolism, smoked menthol is not, because of systemic delivery.[14]

Menthol is not only a flavor additive; it is a potent ligand for transient receptor potential (TRP) cation channels, including TRPM8 (cold-sensitive).[15] In addition, menthol modulates other ion channels, such as nicotinic acetylcholine receptors (nAChRs).[16–18] Because menthol has sensory effects (eg, cooling), it may interact with nicotine and make it much easier to consume.[19] Menthol is preferred by ~30% of US smokers with ~80% of Black smokers consuming menthol cigarettes primarily[20] and it appeals to young inexperienced smokers.[21] There is growing evidence that smokers find harder to quit mentholated cigarettes versus non-mentholated cigarettes.[22] Menthol may serve as a conditioned stimulus by its effects as a sensory stimulant that reinforces the rewarding effects of nicotine.[23]

However, the menthol–nicotine interaction has not been fully clarified yet, particularly, the impact of menthol on oral nicotine consumption and preference remain unclear. A recent report has suggested that menthol can reduce the aversive effects of oral nicotine through TRPM8 receptors dependent sensory mechanisms in mice.[24] For this reason, in the present study, we determined the effects of oral menthol on oral nicotine consumption and preference using the two-bottle free choice paradigm in rats. The effect of oral menthol on plasma nicotine levels was also examined at the end of the study.

Materials and Methods

Animals

The study was performed on total 64 adult (10–12 weeks of age at the beginning of experiment) female and male Sprague Dawley rats (supplied by the Experimental Animals Breeding and Research Center, Uludag University). Animals were housed in conventional cages with 1 rat per cage in a temperature- and humidity-controlled room (21 ± 2°C, 50 ± 5%) on a 12-hour light/dark cycle. The rats were provided free access to food and received their water as drinking solutions from two bottles at all times. The study was approved by the Local Ethics Committee for Animal Experiments, Uludag University (approval number: 2015-12/02).

Drugs and Chemicals

(−)-Menthol was purchased from Sharp Mint Limited (India) and (−)-Nicotine hydrogen tartrate salt [(−)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] was purchased from Sigma-Aldrich (St. Louis, MO). All doses are expressed as the free base of the drug. Menthol and nicotine solutions were prepared by dissolving (−)-menthol (100–1000 mg/L) and/or (-)-nicotine (3 and 20 mg/L) in distilled water containing 5% alcohol (ethanol, Fisher Scientific). All drug solutions were prepared fresh daily.

Two-Bottle Choice Test

The two-bottle free choice paradigm, which allows for free access to nicotine in a drinking solution, has been used to study chronic nicotine exposure in rodents.[25–27] Therefore, this method was used to assess the impact of menthol administration on nicotine intake in male and female rats. The rats were given a choice of water or drug solution using a two-bottle choice procedure. The rats were divided into four equally sized groups for both sexes (n = 8 per group):

- The first group of rats received a nicotine solution versus water. On the first and the second day, the animals received 3 mg/L of nicotine followed by 20 mg/L of nicotine for 14 days.
- The second group of rats received a menthol plus nicotine solution versus water. The effect of menthol on nicotine intake was evaluated by exposing the rats to mentholated nicotine solution at the same nicotine dosing schedule as in the first group. In addition, the rats received 100 mg/L on the first and the second day followed by 1 g/L menthol for 14 days. The range of nicotine and menthol doses were selected based on previous nicotine research with rats.[28,29]
- The third group of rats were given a menthol solution versus water. Rats received on the first and the second day 100 mg/L and followed by 1 g/L menthol for 14 days.
- The final fourth group of rats were exposed to a vehicle (5% ethanol) solution versus water to evaluate the preference to the vehicle that was used in this study.

Tests were performed on individually housed rats who had been acclimated to their respective housing for 1 week and presented with two 250 ml dark blue plastic graded bottles containing metal spouts. One bottle contained tap water, while the other bottle contained the drug solution. During the test, the drug solution and water bottles positions were changed daily to avoid any potential rat bias for bottle placement. All groups were studied under the same experimental conditions. During the period of the test, female and male rats were housed in two separated rooms according to their sex under the same conditions. Daily water and drug intake were measured by reading the volume intake. The reduced volume was replaced with the same solution. The data was recorded every 24 hours as a daily dose (mg/kg/day) and total daily fluid intake was recorded as water and drug intake volume. Nicotine preference was calculated as the volume of nicotine consumed as a percentage of

the total fluid consumed. To minimize the effect of handling-stress on behavior, the rats were weighed every other day and the average mass (in kg) of each rat was used for their daily drug intake dose calculation.

Plasma Nicotine Levels Measurement

To determine plasma nicotine levels in the first and second group of rats, blood samples were drawn by cardiac puncture under light sevoflurane anesthesia at the end of the study on day 16 after the last measured volume of drinking solutions taken between 10 AM to 4 PM.

Drug Extraction

To a 200 µl aliquot of whole blood, 50 µl of internal standard (ISTD) containing 50 ng of nicotine-d4 in methanol was added with mixing. Then 100 µl of 5 M ammonium hydroxide was added to each sample followed by 2 ml methylene chloride. The samples were mixed for 2 minutes and then centrifuged for 5 minutes at 3000 rpm (1811*g) at a temperature of 4°C. The organic layer was transferred to a clean test tube. The aqueous phase was extracted twice more with 2 ml of methylene chloride. The organic phases were combined and 500 µl of 25 mM hydrochloric acid in methanol was added. The samples were then evaporated until dry under a gentle stream of nitrogen. They were reconstituted with 100 µl of mobile phase and placed in auto-sample vials for analysis.

An Agilent technology 1200 series HPLC system (Agilent Technologies, Boeblingen, Germany) with a binary pump, degasser, an auto sampler, a thermostat column compartment, and a UV detector was used. Chromatography was performed on a reversed-phase C-18 column (Agilent Technologies) of 150×3mmi.d. dimensions and 3.5 µm particle size. The mobile phase consisted of 15 mM ammonium formate in water, pH adjusted to 10.5 with TEA (solvent A) and acetonitrile (solvent B). Gradient elution used was: 0–5 minutes from 9% to 10% solvent B; 5–13 minutes from 10% to 13% solvent B; the flow rate was 0.8 mL/minute. The column temperature was set at 25°C.

Statistical Analysis

The data obtained were analyzed using the GraphPad software, version 7.04 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± SEM. A three-way analysis of variance test (ANOVA) with repeated measures (RM) was used to determine the overall interaction of the three factors: time, sex, nicotine/menthol+nicotine treatment (Supplementary Figure 2). In addition, two subsequent two-way RM ANOVA, followed by the Bonferroni post hoc test were used to determine treatment × sex and time × sex interactions. Two-way RM ANOVA, followed by the Bonferroni post hoc correction was used to evaluate impact of menthol on nicotine intake and preference (Figure 1), to test effects of treatments on body weight and total fluid intake (Supplementary Figure 1) in separate sexes, to analyze menthol intake and preference (Figure 2), and to compare preference to the vehicle between male and female rats (Supplementary Figure 3). Ordinary two-way ANOVA, followed by the Bonferroni post hoc correction was used to assess the differences in plasma nicotine levels with sex (male, female) and treatment (nicotine, menthol+nicotine) as factors (Figure 3). Before ANOVA, the data were first assessed for the normality of the residuals and equal variance. Variances were similar between groups and were assessed using either the F-test or the Brown–Forsythe test and the Bartlett's test. All data passed these tests. The p values < .05 were considered significant.

Results

Effects of Drugs on Body Weight Change and Total Fluid Intake

Nicotine, menthol-nicotine combination, menthol and vehicle solutions did not differentially affect body weight gain in male rats ($F_{\text{treatment (3,21)}} = 0.083$, $p = .968$ and $F_{\text{time (7,49)}} = 2.256$, $p < .05$; Supplementary Figure 1a). Similarly, the female groups showed no significant differences in body weight ($F_{\text{treatment (3,21)}} = 3.046$, $p = .06$ and $F_{\text{time (7,49)}} = 58.38$, $p < .05$; Supplementary Figure 1c).

Overall, the average of total fluid intake in male group of rats was 75.9 ± 1.8 (vehicle), 67.5 ± 1.7 (nicotine), 76.2 ± 2.1 (menthol), and 72.6 ± 2.3 ml (menthol plus nicotine). The average of total fluid intake in female group of rats was 61.7 ± 2.2 (vehicle), 65.2 ± 2.7 (nicotine), 63.7 ± 1.9 (menthol), and 58.33 ± 2.1 ml (menthol plus nicotine). Total fluid intake of treatment groups in male ($F_{\text{treatment (3,21)}} = 4.04$, $p < .05$ and $F_{\text{time (15,105)}} = 6.759$, $p < .001$; Supplementary Figure 1b) and female ($F_{\text{treatment (3,21)}} = 1.579$, $p = .2243$ and $F_{\text{time (15,105)}} = 14.41$, $p < .001$; Supplementary Figure 1d) were similar except on certain days. The total fluid intake of the nicotine group was lower than the vehicle group at certain time points (on days 7, 8, 9, 11, and 16) in male rats. Total fluid intake in nicotine group of female rats was higher on day 2 and lower on day 7 than vehicle group.

Effects of Menthol on Nicotine Intake

As seen in Figure 1, menthol addition to nicotine solution substantially increased nicotine intake ($F_{\text{time (15,105)}} = 19.53$, $p < .001$ and $F_{\text{treatment (1,7)}} = 53.19$, $p < .001$; Figure 1a) and preference ($F_{\text{time (15,105)}} = 3.881$, $p < .001$ and $F_{\text{treatment (1,74)}} = 26.88$, $p < .01$; Figure 1b) across most days in the male rats. Menthol significantly increased the nicotine intake in a time-related manner until day 8. The effect of menthol on nicotine intake gradually reduced after that day, but remained significant (Figure 1a). The average nicotine intake in male rats was approximately 0.5 ± 0.05 and 1.4 ± 0.12 mg/kg for nicotine and menthol-nicotine combination, respectively. In contrast to male rats, nicotine intake did not increase in the menthol-nicotine combination group in female rats ($F_{\text{time (15,105)}} = 22.16$, $p < .001$ and $F_{\text{treatment (1,7)}} = 0.0444$, $p = .839$; Figure 1c). Similarly, menthol addition did not induce a distinctive overall change in nicotine preference in female rats. Female rats only showed a modest increase in nicotine preference on days 7, 9, and 13 in mentholated nicotine group ($F_{\text{time (15,105)}} = 4.547$, $p < .001$ and $F_{\text{treatment (1,7)}} = 19.8$, $p < .01$; Figure 1d). The average nicotine intake in female rats was approximately 0.6 ± 0.05 and 0.6 ± 0.03 mg/kg for nicotine and menthol-nicotine combination, respectively.

In order to determine sex differences in menthol's effects, the results shown in Figure 1 were re-evaluated by three-way ANOVA. Significant effects of menthol on nicotine intake were found for all factors (time, sex, and treatment) ($F_{\text{time × sex × treatment (15,448)}} = 4.75$, $p < .001$; Supplementary Figure 2a). Male rats had statistically higher menthol + nicotine consumption on days 4, 6, 7, 8, 9, 10, 11, 12, and 16 than female counter parts. Female rats in nicotine groups had higher nicotine consumption on days 3, 4, and 10 than males (Supplementary Figure 2a). Although there was no significant interaction between the three factors on nicotine preference by menthol ($F_{\text{time × sex × treatment (15,448)}} = 1.203$, $p = .2656$; Supplementary Figure 2b), we found that menthol resulted in a significant time × sex interaction ($F_{\text{time × sex (15,448)}} = 2.749$, $p < .001$) and treatment × sex interaction ($F_{\text{time × sex × treatment (1,448)}} = 94.07$, $p < .001$). Male rats in menthol + nicotine group showed higher preference on days 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, and 16 than female counter parts. On days 7 and 9, male rats in nicotine groups had higher preference than females (Supplementary Figure 2b).

Menthol Intake and Preference in Male and Female Rats

To evaluate menthol intake and preference in rats, third group of animals were given a choice of menthol or water (Figure 2). Female and male rats were exposed to menthol 100 mg/L concentration for 2 days and followed by 1 g/L menthol for 14 days. Two-way ANOVA revealed significant effects on some time points for menthol intake ($F_{time\ (15,105)} = 15.35$, $p < .001$ and $F_{sex\ (1,7)} = 2.276$, $p = .1751$; Figure 2a) and preference ($F_{time\ (15,105)} = 3.342$, $p < .001$ and $F_{sex\ (1,7)} = 4.5$, $p = .071$; Figure 2b) in female and male rats. While male rats drank

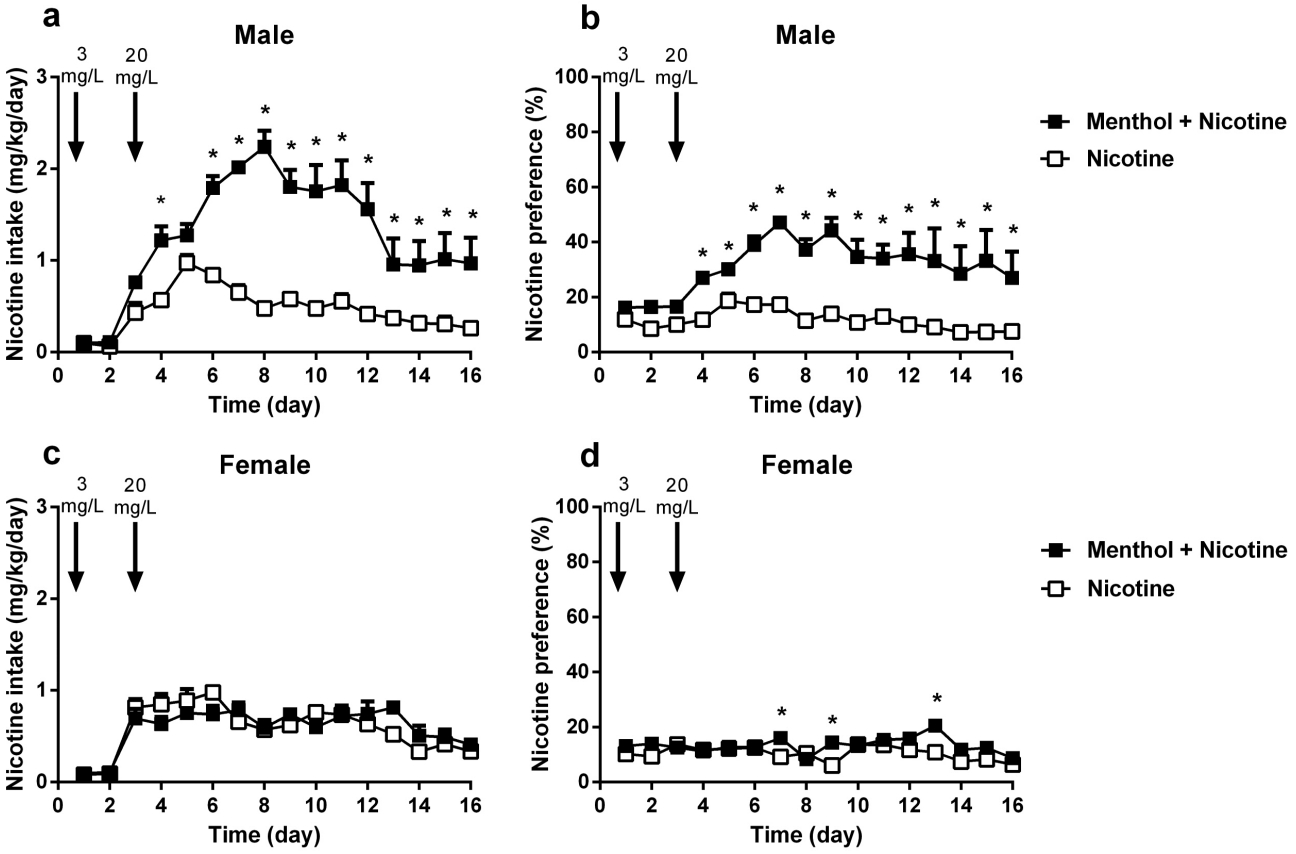

Figure 1. Effects of menthol on oral nicotine intake (a, c) and preference (b, d) in rats. Graphs in (a) and (b) indicate the results from male rats where (c) and (d) from female rats. Rats received either mentholated nicotine solution and water or nicotine solution and water as in two-bottle choice paradigm. Nicotine group received 3 mg/L nicotine in their drug solution at first and second days, and followed by 20 mg/L nicotine concentration for 14 days. Effect of menthol on nicotine intake was evaluated by exposing the rats to mentholated nicotine solution with same doses in a separate cohort of rats. Mentholated nicotine group received 100 mg/L menthol plus 3 mg/L nicotine at first and second days and followed by 1 g/L menthol plus 20 mg/L nicotine for 14 days. Each time point shows the average consumption of day. Data are presented as the mean ± SEM of eight rats. *$p < .05$.

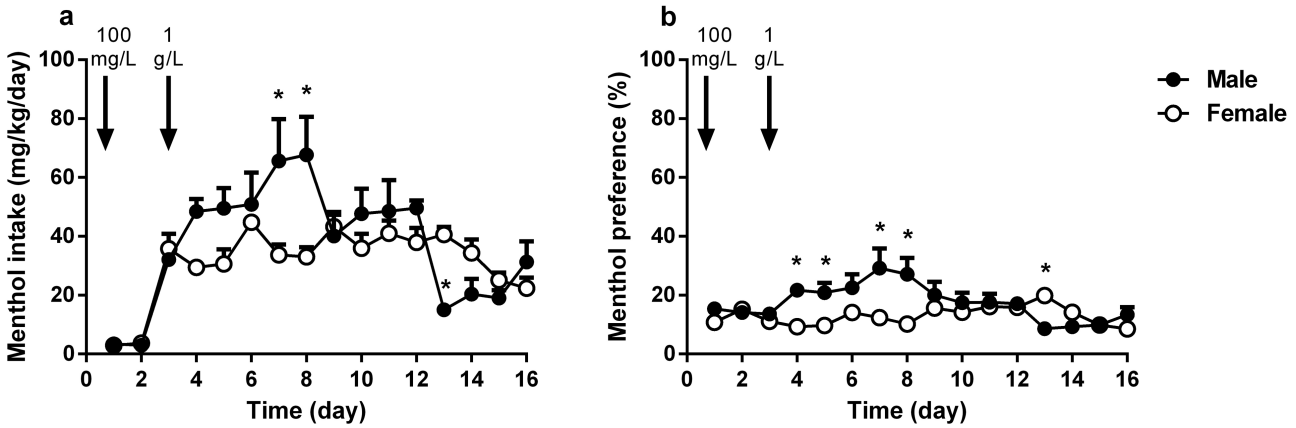

Figure 2. Menthol intake (a) and preference (b) in male and female rats. Animals received menthol solution and water as in two-bottle choice paradigm. Menthol was given as 100 mg/L concentration at first and second days and followed by 1 g/L concentration for 14 days. Data are presented as the mean ± SEM of eight rats. *$p < .05$.

more menthol on day 7 ($p < .001$) and day 8 ($p < .001$), female rats drank more on day 13 ($p < .05$). Similarly, male rats showed higher preference on day 4 ($p < .05$), day 5 ($p < .05$), day 7 ($p < .001$), day 8 ($p < .001$) while female rats preferred menthol more than the males on day 13 ($p < .05$). Overall, when rats were given menthol solution (1 g/L) or water, average menthol intake was approximately 41.8 ± 4.4 mg/kg for males and 34.8 ± 1.7 mg/kg for females ($p = .149$). Furthermore, average menthol preference was 17.7% ± 1.7 for male and 12.9% ± 0.9 for female rats from day 3 to 16 ($p = .025$).

In order to evaluate whether the vehicle of menthol alters consumption, a fourth group of animals were exposed to 5% ethanol (vehicle) or water in a two-bottle choice test and the preference of vehicle was evaluated. As seen in Supplementary Figure 3, no significant difference on preference for the vehicle was seen between male and female rats ($F_{time\ (15,105)} = 1.688$, $p = .064$ and $F_{sex\ (1,7)} = 0.7714$, $p = .408$).

Effects of Menthol on Plasma Nicotine Levels

In order to determine if menthol addition alters plasma nicotine levels, blood was collected at the end of the experiment. Two-way ANOVA showed that there was no significant effects on plasma nicotine levels between treatments and sexes ($F_{treatment\ (1,25)} = 0.436$, $p = .515$ and $F_{sex\ (1,25)} = 2.183$, $p = .152$; Figure 3). As seen in Figure 3, we observed a 1.5-fold increase in nicotine plasma levels in the menthol plus nicotine-treated male rats when compared with the nicotine alone group. However, this increase did not reach statistically significant levels ($p = .0861$). No differences were detected in nicotine plasma levels of female rats between mentholated and non-mentholated nicotine groups ($p = .1896$).

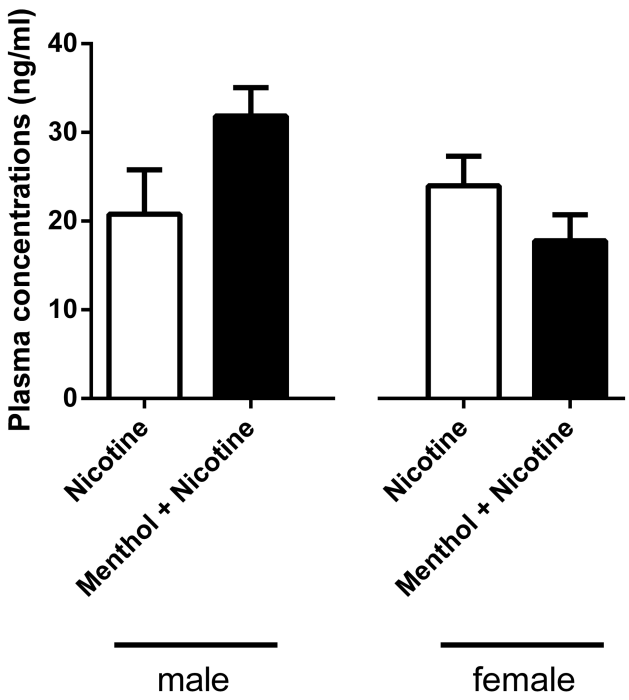

Figure 3. Impact of menthol on plasma nicotine levels. Blood samples were drawn by cardiac puncture at the end of the study on day 16 after last measurement of volume of drinking solutions. Nicotine group received 3 mg/L nicotine in their drug solution at first and second days, and followed by 20 mg/L nicotine concentration for 14 days. Mentholated nicotine group received 100 mg/L menthol plus 3 mg/L nicotine at first and second days and followed by 1 g/L menthol plus 20 mg/L nicotine for 14 days. Data are presented as the mean ± SEM of seven to eight rats.

Discussion

This study examined the effect of menthol on oral nicotine intake and preference in adult rats. Using a free choice oral nicotine consumption procedure, our results showed that menthol addition to nicotine solution increased nicotine intake and preference in male but not female rats without a significant effect on total fluid intake or body weight changes.

When rodents are exposed to a voluntary choice of water or nicotine solution, individual consumption varies with age and sex also possibly influencing consumption.[26,28,30–34] As seen in Supplementary Figure 2a and b, female rats showed higher amount of nicotine intake than male rats on certain days (3, 4 and 10), but the preference ratio was not different between the groups. Overall, 20 mg/L concentration of nicotine in our studies showed a modest stable drinking behavior and intake level in each sex which is consistent with previous reports in adult rats.[28,33] This dose is also below nicotine concentrations (>50 mg/L) reported to decrease ingestive responses and to increase aversive type taste reactivity responses in rats.[32]

As seen in Figure 1, the addition of menthol to nicotine solution resulted in a significant increase of nicotine consumption in males but not in females. Consistent with our results, a recent study showed that menthol enhanced the reinforcing actions of nicotine. Systemic menthol pretreatment resulted in a leftward shift of the inverted U-shaped nicotine dose-response curve on intravenous nicotine self-administration in male rats.[35] Additionally, oral menthol cue facilitated intravenous nicotine self-administration via its cooling effect in adolescent female rats.[29] It has recently been reported that menthol (100 µg/mL) can reduce the aversive effects of oral nicotine (200 µg/mL) in two-bottle choice paradigm in male mice through TRPM8 receptor involvement.[24] Moreover, oral 0.005% menthol reversed nicotine (50 and 100 µg/mL) aversion in male rats using a modified 1 hour two-bottle choice test.[36] While these reports support the hypothesis that menthol facilitates nicotine reinforcement and increases nicotine consumption, sex differences were not studied. Therefore, the effect of menthol on nicotine intake in both sexes were compared in this study under the same experimental conditions and it was found that menthol enhanced nicotine consumption in male but not female rats. Three-way ANOVA revealed a significant effect of menthol on nicotine intake in time, sex, and treatment factors. Male rats significantly had higher mentholated nicotine consumption than female rats (Supplementary Figure 2). In a recent study, mice were given nicotine, menthol, or mentholated nicotine solution as their sole source of fluid.[37] Consistent with our results, menthol addition induced greater consumption in adult male mice but not in female mice. Sex- and age-dependent differences were found in menthol's effect.[37] Male and female rodents may exhibit different sensitivities to noxious and cooling sensitivity. For example, prolactin has been found to alter TRPM8 channel activity during inflammatory noxious stimulus.[38] Recent human data have also shown gender differences in menthol-induced enhancement in the rate of brain nicotine accumulation during mentholated cigarettes smoking in men but not women compared to non-mentholated ones.[39] However, epidemiological studies have emphasized that women smokers prefer mentholated cigarettes more than men.[40,41] Different mechanisms may explain the contradictions between oral dosing paradigm and inhalation.

Several distinct mechanisms may underlie menthol's effects in male rats. Sensory effects of menthol may lead to an increase in nicotine intake through a "cooling" effect and/or masking the taste

of nicotine. The sensory effects of menthol may partially explain the results that were observed on nicotine consumption. Male rats showed higher menthol preference (average increase of 1.35-fold) and consumption (average increase of 1.2-fold) on certain days than female rats (Figure 2). However, the magnitude of this enhancement was modest and much less substantial than the intake and preference found in menthol-nicotine combination group (preference average increase of 2.83-fold and consumption average increase of 2.90-fold). Menthol may also work by altering nicotine pharmacokinetics and metabolism. Menthol has been shown to increase the transbuccal permeability and penetration of nicotine.[42–44] Menthol has been found to either slow or not alter the nicotine metabolism after mentholated cigarette smoking or nicotine exposure in humans[45,46] and rodents.[47,48] In addition, Ghazi et al.[49] reported that effect of oral mint drinks increased urine nicotine/cotinine ratio in adult Jordanian male volunteers, suggesting an impact on decreasing nicotine metabolism to cotinine. Even though one possible mechanism underlying effects of oral menthol seen in our studies can result from slowed nicotine metabolism by menthol in rats, different enzymes mediate nicotine metabolism in humans. The enzymes CYP2B1/2, which primarily metabolize nicotine to cotinine in rats[50] are different from CYP2A6 that metabolizes nicotine in humans.[51] In addition, sex hormones may influence nicotine metabolism. For example, nicotine metabolism has been found faster in women than in men.[52] However, female rats generally have three- to fivefold lower rate of hepatic drug metabolism than male rats.[53] Nicotine's half-life is longer in female versus male rats.[53] In this study, similar plasma nicotine levels were found in female and male rats with the current dose of nicotine. On the other hand, menthol slightly (~1.5-fold) increased plasma nicotine levels compared to nicotine alone in male rats, which is consistent with previous data obtained from male mice and rats.[47,48] Nonetheless, oral menthol may have different effects on nicotine metabolism in rats compared to human.

Menthol may have differentially impacted the underlying neurobiology of nicotine dependence in male and female rats. Recently, data have emerged to suggest that menthol allosterically modulates nAChR function.[16–18] Menthol is a negative allosteric modulator of α4β2 nAChRs[16] and α3β4 nAChRs.[18] Besides, chronic administration of menthol alone or combination with nicotine is sufficient to upregulate brain nAChRs involved in reward in male mice.[48,54,55] Although some reports showed that menthol and nicotine by itself or combination induce nAChRs upregulation without sex differences in mice,[54,55] other report showed that nicotine administration for 10 days induces greater nAChR upregulation in male mice compared to females.[56] Similarly, male but not female human smokers upregulate brain β2*-nicotinic receptors compared to nonsmokers.[57] Menthol may also influence dopamine signaling in central nervous system. Menthol enhances nicotine reward by increasing nicotine-induced TH+/dopamine-neuron firing frequency.[55] Menthol itself,[54] but also menthol+nicotine combination upregulates α4α6* nAChR number and function on midbrain dopamine neurons (menthol+nicotine more than nicotine alone).[55] Therefore, menthol may alter nicotine-induced dopamine signaling in the brain. However, when male rats were given intraoral menthol (0.005%), phasic dopamine concentrations remained similar to water control groups.[36] The concentration of menthol may play a role in different dopamine signaling outcome by menthol. Moreover, GABAergic neurons play an important role in nicotine's effects.[58] Dopaminergic neuron activity in the ventral tegmental area is regulated by glutamatergic and GABAergic interneurons, where nAChRs modulate synaptic activity.[59] It has been shown that menthol enhances tonic inhibition in rat hippocampal cultures, which is mediated by slowly desensitizing $GABA_A$ receptors.[60] Since menthol is a positive allosteric modulator of $GABA_A$ receptors,[61] the enhanced nicotine intake observed in male rats treated with mentholated nicotine may be due to GABAergic mechanisms.

Lastly, TRPM8, the primary menthol sensor,[62] may show a possible mechanism underlying menthol's effects on oral nicotine intake. The expression of TRPM8 ion channels on the trigeminal neurons innervating the oral cavity and tongue are similar in humans and rodents. It has been suggested that these neurons activate inhibitory circuits in the trigeminal nucleus that depress the input from nicotine-activated nociceptors and lead to a reduction of oral irritation and pain.[24]

In summary, menthol increases nicotine drinking behavior in male but not in female rats. Sensory, central, and/or metabolism mechanisms of menthol may be the underlying reasons for preference in menthol-containing oral smokeless tobacco products.

Supplementary Material

Supplementary data are available at *Nicotine and Tobacco Research* online.

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Declaration of Interests

None of the other authors declared a conflict of interest. RFT has consulted for Apotex and Quinn-Emmanual on unrelated matters.

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