

## Original Article

# Effects of menthol-flavored substances at the cellular level on oral mucosal sites

Nanda Prasad<sup>1</sup>, Sarvesh Vijay<sup>1</sup>, A. Yashwanth Reddy<sup>1</sup>, S. Nonitha<sup>1</sup>

<sup>1</sup>Department of Oral and Maxillofacial Pathology, Sri Rajiv Gandhi College of Dental Sciences and Hospital, Bengaluru, Karnataka, India

## ABSTRACT

**Background:** The purpose of this study was to determine the effects of menthol-flavored substances at the cellular level in different mucosal sites of the oral cavity and to compare the cellular changes between individuals without the habit of chewing menthol-flavored substances and individuals with the habit.

**Materials and Methods:** This was an experimental cytology study including a total of 500 individuals belonging to the age group of 18–45 years based on the inclusion or exclusion criteria. The selected participants were divided into two groups of 250 participants each, based on participants not having the habit of chewing menthol-flavored substances (Group I) and participants having the habit of chewing menthol-flavored substances (Group II). Cytological smears were taken by gently scraping the mucosal surfaces in different sites of the oral mucosa using a wooden spatula and stained with Papanicolaou, analyzed under microscope for any cellular changes. The results were tabulated and statistically analyzed using Chi-square test and Fisher's exact test.  $P < 0.05$  was considered statistically significant.

**Results:** Micronuclei seen in all the participants belonging to group with the habit of chewing menthol-flavored substances with a  $P < 0.001$  which was considered highly significant. Alteration in the nuclear-cytoplasmic ratio was also seen  $P = 0.001$ , which showed significant at 1% significance level.

**Conclusion:** Participants with habit of chewing menthol-flavored substances showed the presence of micronuclei and slight alteration in the nuclear-cytoplasmic ratio, which could be directly related to genotoxicity and cell damage.

**Key Words:** Cell death, chewing, menthol

Received: March 2017  
Accepted: January 2018

Address for correspondence:  
Dr. Sarvesh Vijay,  
Department of Oral and  
Maxillofacial Pathology,  
Sri Rajiv Gandhi College  
of Dental Sciences and  
Hospital, Cholanagar,  
Hebbal, Bengaluru - 560 032,  
Karnataka, India.  
E-mail: sarvesh.rdc@gmail.  
com

## INTRODUCTION

The name “spear” mint derives from the pointed leaf tips of the plant. Mint descends from the Latin word *mentha*, which is rooted in the Greek word *minthe*, personified in Greek mythology as *Minthe*. There are about 26 species of mint. All of them considered as the most fragrant plants with a pleasant taste and many healing properties. The main active substances

contained in the essential oil of peppermint are menthol (alcohol), the menthone (ketone), and tannins. Its leaves contain Vitamins A and C, niacin (Vitamin B3), magnesium, and iron.<sup>[1]</sup>

Menthol is one of the monocyclic terpenes that occur naturally in more than 100 essential oils, which includes spearmint and peppermint. The characteristic

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**For reprints contact:** reprints@medknow.com

**How to cite this article:** Prasad N, Vijay S, Reddy AY, Nonitha S. Effects of menthol-flavored substances at the cellular level on oral mucosal sites. Dent Res J 2019;16:7-11.

Access this article online



Website: [www.drj.ir](http://www.drj.ir)  
[www.drjjournal.net](http://www.drjjournal.net)  
[www.ncbi.nlm.nih.gov/pmc/journals/1480](http://www.ncbi.nlm.nih.gov/pmc/journals/1480)

minty aroma and cooling qualities of synthetic d-menthol and the natural l-menthol isomer have resulted in its use in a variety of commercial food and pharmaceutical products.<sup>[2,3]</sup> The chewing of nonfood items for pleasure has a long history.<sup>[4]</sup> Tree resins were chewed by the ancient Egyptians, the Mayan Indians, and the early American Indians. The first commercial chewing gum, State of Maine Pure Spruce Gum, appeared in 1848.<sup>[5]</sup> During chewing, the oral cavity functions such as a bellows, forcing volatile flavor compounds into the exhaled air to the nasal compartment.<sup>[6]</sup> Accordingly, that flavor release from chewing gum is predominantly governed by chewing frequency, although other oral functions, such as masseter muscle activity, chewing force, and saliva flow rate, may also play a role.<sup>[7]</sup> Menthol appears to alter cell membranes, and the findings of animal studies have suggested, that there are changes in cell membrane integrity and it does appear, however, that menthol alters cell permeability too.<sup>[8,9]</sup> Menthol acts as a coolant and a mild local anesthetic.<sup>[10]</sup> One of the more immediate cellular effects of menthol on cell membranes is that of cell death (cytotoxicity).<sup>[11]</sup> Menthol has been shown to be toxic *in vitro* biologic model systems in normal tissue,<sup>[12]</sup> it inhibits fatty acid-induced (receptor-mediated) cell respiration in brown adipose tissue and increased cellular respiration rate and osmotic swelling suggesting deterioration of biologic membranes in mitochondria.<sup>[13]</sup> Growing evidence has shown that menthol can induce mitochondrial membrane depolarization<sup>[14]</sup> through the transient receptor potential melastatin family member 8 (TRPM8) channel in cells of the human bladder cancer cell line T24 resulting in cell death.<sup>[15]</sup> Since the 1920s, menthol has been added to cigarettes and used as a characterizing flavor. However, its use in chewing gums came after 1950s.<sup>[16]</sup> Among other effects, menthol vapor can modulate sensitivity to chemical irritation in the upper airways in humans.<sup>[17]</sup> Inhaled menthol also exerts complex olfactory and sensory effects by interacting with olfactory and somatosensory neurons and respiratory tissues.<sup>[18]</sup> It is puzzling, in the light of increased awareness, that more studies such as ours have not been undertaken. However, few studies have been done in English literature so far to evaluate toxicity and cellular effects of menthol. Gaworski *et al.* exposed rats to menthol cigarette smoke through nose inhalation for 1 h a day, 5 days/week for 13 weeks. In their study, they concluded that exposure to menthol cigarette smoke produced reduced body weights

and histopathological changes including epithelial hyperplasia and/or squamous metaplasia in the nasal passages, trachea, larynx, lungs, and bronchi. Olfactory epithelial degeneration was also observed in these cases.<sup>[2]</sup> As a parallel to the findings by Gaworski *et al.*, Alakayak and Knall found that the gap junctions between the cells were “loosened” up and integrity was lost as a result of effect of tobacco smoke effects on (transepithelial electrical resistance the tight gap junctions between the human bronchial epithelial cells).<sup>[15]</sup> To the best of our knowledge, no study in English literature has been done so far to study the effect of chewing menthol-flavored substances. Therefore, this study was undertaken to see the effect of the use of menthol-flavored substances at the cellular level of the mucosal sites of oral cavity.

## MATERIALS AND METHODS

This comparative experimental cytology study was conducted with approval from Institutional and Ethical committee (SRGCDS/2015/502) in the department of oral and maxillofacial pathology in our institution from February 2016 to April 2016. Informed consent was obtained from all individuals before the initiation of the study. This study has two groups. The control group (Group I) consists of individuals not having the habit of chewing menthol-flavored substances, and the study group (Group II) consists of individuals having the habit of chewing menthol-flavored substances. A total of 500 participants were evaluated, out of which 250 participants have the habit of chewing only menthol-flavored substances for more than 5 years while the remaining 250 participants do not have the habit of chewing menthol-flavored substances. Participants in the age group between 18 and 45 years in each group were evaluated. Inclusion criteria included participants with the habit of chewing 5 or more than 5 menthol-flavored substances per day for more than 5 years. For this, the participants reporting to our outpatient department were asked randomly whether they had this habit and took their consent. Once conformed they were asked to sign consent letter whereas the exclusion criteria included participants having the habit of chewing other than menthol-flavored substances. Participants having the habit of chewing less than 5 menthol-flavored substances and participants having any systemic diseases, smoking habits, and other oral manifestations such as proliferative, traumatic, or

immune-mediated epithelial lesions (e.g., papilloma, aphthous ulcer, lichen planus, and traumatic ulcer) were excluded from our study. Cytological smears were taken by gently scraping the mucosal surfaces in different sites of the oral mucosa, using a wooden spatula supplied from Asian hobby crafts, New Delhi, India. For the preparation of smears, clean, fresh, dry glass slides (Blue star micro slides, supplied from Polar industrial corporation Mumbai, India) were used. The material from the wooden spatula was spread on the middle third of clean dried glass slides. The smears were spread over a large area, preventing the clumping of cells. The prepared slides were immediately sprayed with Biofix spray microanatomy fixative supplied from Biolab Diagnostics Pvt., Ltd., Tarapur, Maharashtra, India, to ensure proper fixation. The smears were stained by using the rapid Papanicolaou (PAP) stain supplied from Biolab Diagnostics Pvt., Ltd., Tarapur, Maharashtra, India.

#### Papanicolaou stain procedure

1. Smears were hydrated by pouring few drops of distilled water on the slide for 1–3 min
2. Excess water was blot out from the slide, and nuclear stain was poured on the slide for 45–60 s
3. On the same slide, 3 drops of buffer solution (Scott's buffer solution) were added and kept for 30–40 s
4. After draining the buffer solution, dehydrant was poured with two changes each for 30 s
5. After discarding dehydrant, working solution (2A + 2B) was poured and kept for 45 s
6. After draining the above solution, dehydrant was poured with two changes each for 30 s followed by xylene for 30 s and mounted with coverslip using dibutyl phthalate xylene.

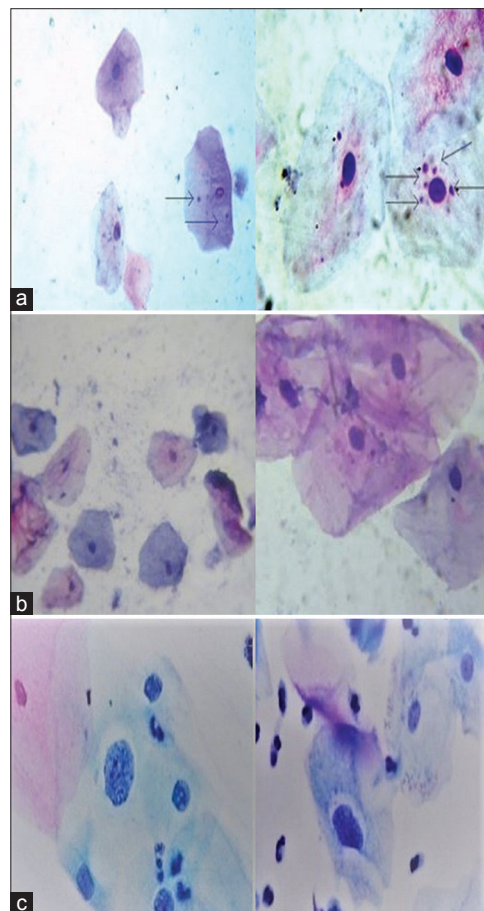
Stained slides from both the groups were then analyzed and verified by five different oral pathologists who were blinded to eliminate bias under the microscope (Lawrence and Mayo, London) who were reporting on the presence of micronuclei and alteration in the nucleus-cytoplasmic ratio to determine the effects of menthol-flavored substances at the cellular level in different mucosal sites of the oral cavity, and the observatory findings were subjected to appropriate statistical analysis. Interobserver reliability for all 5 observers was tested. The Cronbach's alpha test proved that there was good interobserver reliability (>0.7) among all the five different oral pathologists.

#### Statistical analysis

All the obtained data were entered in an Excel Spreadsheet. A comparative study was done using Chi-square test and Fisher's exact test between the participants not having the habit of chewing menthol-flavored substances and participants having the habit of chewing menthol flavored substances. The values were tabulated for comparison purpose.  $P < 0.05$  in Chi-square test and Fisher's exact test was accepted as indicating statistical significance.

## RESULTS

On observing the cytological smears, micronuclei was seen in all the 250 participants belonging to study group and in none of the participants in the control group [Figure 1a and b and Table 1] with a  $P < 0.001$  which was considered highly significant. Alteration in the nuclear-cytoplasmic ratio was also seen in 90



**Figure 1:** (a) Photomicrograph showing the presence of micronuclei in the study group. (Papanicolaou  $\times 10$ , Papanicolaou  $\times 40$ ). (b) Exfoliated cells in control group (Papanicolaou  $\times 10$ , Papanicolaou  $\times 40$ ). (c) Altered nuclear-cytoplasmic ratio in the study group (Papanicolaou  $\times 10$ , Papanicolaou  $\times 40$ ).



participants out of 250 participants in study group with a  $P = 0.001$ , which showed significant at 1% significance level [Figure 1c and Table 2].

## DISCUSSION

This study examined the consequences of using menthol-flavored substances at the cellular level of the mucosal sites of oral cavity and hence the potential for increased cytotoxicity. Menthol is one of the monocyclic terpenes that occur naturally in over 100 essential oils, which includes spearmint and peppermint. The characteristic minty aroma, cooling qualities, and mild local anesthetic properties have resulted in its use in a variety of commercial food and pharmaceutical products. The world market for chewing gum is estimated to be around 560,000 tons/year, and around 374 billion pieces of chewing gum are sold worldwide every year, representing 187 billion of gum-chewing if each piece of gum is chewed for 30 min. Chewing gum can thus be expected to have an influence on oral health.<sup>[4]</sup> One of the more immediate cellular effects of menthol on cell membranes is that of cell death (cytotoxicity). In 1997, Gaworski *et al.* exposed rats to menthol or nonmenthol cigarette smoke through nose-inhalation for 1 h a day, 5 days/week for 13 weeks and concluded that smoke produced reduced body weights and histopathological changes including epithelial hyperplasia and/or squamous metaplasia in the nasal passages, trachea, larynx,

lungs, and bronchi olfactory epithelial degeneration was also observed.<sup>[2]</sup> In 2006, Azzi *et al.* conducted a study on permeation and reservoir formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[a] pyrene across porcine esophageal tissue in the presence of ethanol and menthol and concluded that menthol alters cell permeability.<sup>[8]</sup> Alakayak and Knall 2008 conducted a study evaluating tobacco smoke effects on transepithelial electrical resistance and concluded that both menthol and nonmenthol smoke reduce transepithelial electrical resistance.<sup>[15]</sup> Wise *et al.* 2011 stated that menthol vapor can modulate sensitivity to chemical irritation in the upper airways in humans.<sup>[17]</sup> Similarly, many studies carried out over the past 20 years have shown that menthol is capable of increasing both the transdermal and transbuccal penetration of dideoxycytidine, propofol, propranolol, and ofloxacin. Menthol's characteristic cooling sensation is due, in part, to the activation of sensory neurons generally termed transient receptor potential channels, in particular, TRPM8 and transient receptor potential subfamily A, member 1. Menthol acts on TRPM8 receptors by rapidly increasing intracellular calcium and mobilizing calcium flux through the channels to induce cold response signals at the application site. Aside from its cold-inducing sensation capabilities, menthol exhibits cytotoxic effects in cancer cells, induces reduction in malignant cell growth, and engages in synergistic excitation of gamma-aminobutyric receptors and sodium ion channels resulting in analgesia.<sup>[19,20]</sup> Menthol, likely due to its effects on cold-sensing peripheral sensory neurons, is known to inhibit the sensation of irritation elicited by respiratory irritants.<sup>[21]</sup> Some studies demonstrate that menthol attenuates signaling through human  $\alpha_3 \beta_4$  nAChRs. Menthol also acts as a competitive inhibitor on the specific binding of [3H] PN 200-110 and [3H] nitrendipine, dihydropyridine class antagonists of L-type  $Ca^{2+}$  channels in cardiac and smooth muscles and neuronal tissue.<sup>[22]</sup> A recent study by Pezzoli *et al.* 2014<sup>[23]</sup> in mouse cortical neurons reported that menthol (250  $\mu$ M) dampens the generation of action potentials in a time- and voltage-dependent manner in TRPM8 knock-out mice and in the presence of a TRPM8 blocker. On reviewing English literature, we found that so far no study has been done to evaluate the effect of chewing menthol-flavored substances on the mucosal sites of the oral cavity. The parameters described in our study shows a higher frequency of micronuclei associated with participants who are in

**Table 1: Presence of micronuclei in various study groups**

Group	Parameter 1 (micronuclei)		Total
	Micronuclei		
	Absent (%)	Present (%)	
Group I	250 (100)	-	250
Group II	-	250 (100)	250
Total	250	250	500

$\chi^2=50.000$ ;  $df=1$ ;  $P<0.001$ ; highly significant

**Table 2: Alteration in nucleus-cytoplasmic ratio in various study groups**

Group	Parameter 2 (nucleus-cytoplasmic ratio)		Total
	Nucleus-cytoplasmic ratio		
	Normal (%)	Altered (%)	
Group I	250 (100)	-	250
Group II	160 (64)	90 (36)	250
Total	410	90	500

$\chi^2=10.976$ ;  $df=1$ ;  $P=0.001$ ; significant at 1% significance level

the habit of chewing menthol-flavored substances as compared to participants who do not have the habit of chewing menthol-flavored substances. A slight alteration in the nucleus-cytoplasmic ratio was also observed in participants having the habit of chewing menthol-flavored substances which may indicate short-term cytotoxicity and cell damage.

## CONCLUSION

To conclude, participants with habit of chewing menthol-flavored substances (Group II) showed the presence of micronuclei and slight alteration in the nuclear-cytoplasmic ratio, which could be directly related to genotoxicity and cell damage. Although we have significant positive results in our study, further research in this area is expected with larger sample size and multiple observers where the cytological smears can be subjected to all stains, to consider menthol-flavored substances as a cytotoxic agent.

## Financial support and sponsorship

Nil.

## Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

## REFERENCES

- Giovino GA, Sidney S, Gfroerer JC, O'Malley PM, Allen JA, Richter PA, *et al.* Epidemiology of menthol cigarette use. *Nicotine Tob Res* 2004;6:67-81.
- Gaworski CL, Dozier MM, Gerhart JM, Rajendran N, Brennecke LH, Aranyi C, *et al.* 13-week inhalation toxicity study of menthol cigarette smoke. *Food Chem Toxicol* 1997;35:683-92.
- Ahijevych K, Garrett BE. The role of menthol in cigarettes as a reinforcer of smoking behavior. *Nicotine Tob Res* 2010;12 Suppl 2:S110-6.
- Amato A, Liotta R, Mulè F. Effects of menthol on circular smooth muscle of human colon: Analysis of the mechanism of action. *Eur J Pharmacol* 2014;740:295-301.
- Imfeld T. Chewing gum – Facts and fiction: A review of gum-chewing and oral health. *Crit Rev Oral Biol Med* 1999;10:405-19.
- Ashoor A, Nordman JC, Veltri D, Yang KH, Al Kury L, Shuba Y, *et al.* Menthol binding and inhibition of  $\alpha 7$ -nicotinic acetylcholine receptors. *PLoS One* 2013;8:e67674.
- Haahr AM, Bardow A, Thomsen CE, Jensen SB, Nauntofte B, Bakke M, *et al.* Release of peppermint flavour compounds from chewing gum: Effect of oral functions. *Physiol Behav* 2004;82:531-40.
- Azzi C, Zhang J, Purdon CH, Chapman JM, Nitcheva D, Hebert JR, *et al.* Permeation and reservoir formation of 4-(methylnitrosamino)-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* 2006;27:137-45.
- Hatem S, Attal N, Willer JC, Bouhassira D. Psychophysical study of the effects of topical application of menthol in healthy volunteers. *Pain* 2006;122:190-6.
- Clark PI, Gardiner PS, Djordjevic MV, Leischow SJ, Robinson RG. Menthol cigarettes: Setting the research agenda. *Nicotine Tob Res* 2004;6 Suppl 1:S5-9.
- Faridi U, Sisodia BS, Shukla AK, Shukla RK, Darokar MP, Dwivedi UN, *et al.* Proteomics indicates modulation of tubulin polymerization by L-menthol inhibiting human epithelial colorectal adenocarcinoma cell proliferation. *Proteomics* 2011;11:2115-9.
- Kim SH, Nam JH, Park EJ, Kim BJ, Kim SJ, So I, *et al.* Menthol regulates TRPM8-independent processes in PC-3 prostate cancer cells. *Biochim Biophys Acta* 2009;1792:33-8.
- Li Q, Wang X, Yang Z, Wang B, Li S. Menthol induces cell death via the TRPM8 channel in the human bladder cancer cell line T24. *Oncology* 2009;77:335-41.
- Schneeberger EE, Lynch RD. The tight junction: A multifunctional complex. *Am J Physiol Cell Physiol* 2004;286:C1213-28.
- Alakayak J, Knall C. Mentholated and non-mentholated cigarettes alter trans-epithelial electrical resistance of calu-3 human bronchial epithelial cells. *Ethn Dis* 2008;18:S145-6.
- Wang J, Roethig HJ, Appleton S, Werley M, Muhammad-Kah R, Mendes P, *et al.* The effect of menthol containing cigarettes on adult smokers' exposure to nicotine and carbon monoxide. *Regul Toxicol Pharmacol* 2010;57:24-30.
- Wise PM, Preti G, Eades J, Wysocki CJ. The effect of menthol vapor on nasal sensitivity to chemical irritation. *Nicotine Tob Res* 2011;13:989-97.
- Willis DN, Liu B, Ha MA, Jordt SE, Morris JB. Menthol attenuates respiratory irritation responses to multiple cigarette smoke irritants. *FASEB J* 2011;25:4434-44.
- Farco JA, Grundmann O. Menthol – Pharmacology of an important naturally medicinal “cool”. *Mini Rev Med Chem* 2013;13:124-31.
- Journigan VB, Zaveri NT. TRPM8 ion channel ligands for new therapeutic applications and as probes to study menthol pharmacology. *Life Sci* 2013;92:425-37.
- Ha MA, Smith GJ, Cichocki JA, Fan L, Liu YS, Caceres AI, *et al.* Menthol attenuates respiratory irritation and elevates blood cotinine in cigarette smoke exposed mice. *PLoS One* 2015;10:e0117128.
- Ton HT, Smart AE, Aguilar BL, Olson TT, Kellar KJ, Ahern GP, *et al.* Menthol enhances the desensitization of human  $\alpha 3\beta 4$  nicotinic acetylcholine receptors. *Mol Pharmacol* 2015;88:256-64.
- Pezzoli M, Elhamedani A, Camacho S, Meystre J, González SM, le Coutre J, *et al.* Dampened neural activity and abolition of epileptic-like activity in cortical slices by active ingredients of spices. *Sci Rep* 2014;4:6825.