Journal of Oral Biology and Craniofacial Research 13 (2023) 796–800

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Nicotine mediated epithelial modulations: An *in-vitro* evidence

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ARTICLE INFO

Keywords:
Nicotine
Oral epithelial cells
Proliferation
Cell-cycle distribution
PI3K/MAPK pathway activation

ABSTRACT

Introduction: Nicotine, the main ingredient in tobacco, acts as a key alkaloid of nearly all tobacco products and has been demonstrated to facilitate tumorigenesis and accelerate metastasis. Further traditional tobacco products have shown to give systemic oral effects such as vasoconstriction, inflammation, and delayed wound healing, however; none of the reports have confirmed the significant knowledge of oral sequel of the effect of nicotine on oral epithelial cells. So, the current study aimed to investigate the effect of nicotine on epithelial transformation to a malignant state.

Material & methods: Through *in-vitro* experiments, the effects of nicotine on epithelial cells obtained from nicotine never exposed buccal mucosa were analyzed using total count and viability test, proliferation assay, cell cycle distribution assay, and PI3K/MAPK dual pathway activation assay.

Result & conclusion: MTT assay demonstrated that the proliferation of epithelial cells takes place at a 150 mM concentration of nicotine. Further, we identified the significantly increased cell count and viability in nicotine-exposed cells. Further, cell cycle distribution assay results demonstrated that nicotine forced the epithelial cells to enter the first growth phase. The same influence of nicotine was observed on the PI3K/MAPK dual pathway activation assay where a greater number of nicotine exposed cells showed dual pathway activation. In conclusion, the current study determined the potential mechanism of action of nicotine on oral epithelial cell proliferation through activating the oncogenic pathway. This may help to develop novel therapeutic strategies for the prevention of malignant transformation from smokeless tobacco-caused oral cancer.

1. Introduction

As per new global cancer statistics 2020, the overall cancer burden has risen to 19.3 million new cancer cases and 10 million cancer deaths all over the globe.[1] Out of this, 50 % of all cases and 58.3 % of cancer deaths are estimated to occur in Asian countries in 2020. In India, cancer of the oral cavity, majorly oral squamous cell carcinoma (OSCC) is the second leading malignancy with a 10.4 % incidence rate after cancer of the breast.[2] According to The Gujarat Cancer & Research Institute (GCRI) hospital-based registry, cancer of the oral cavity is the top leading malignancy amongst men with an incidence rate of 35.17 % while in the female it is the third leading cause of death with an incidence rate of 13.09 %. Such a high prevalence of oral cancer could be attributable to frequent exposure of oral mucosal lining to either combustible or smokeless tobacco, ultimately leading to the development of possibly malignant neoplasms that can histologically exhibit different degrees of several epithelial dysplastic changes.[3]

These oral epithelial dysplasias are transformed to OSCC with a quite high rate of 0.13 %–17.5 %, which makes it unpredictable.[4] Despite upgraded treatment modalities, the 5-year survival rate remains poor at around 50 %.[5] Hence, recognizing the early trigger of malignant transformation is decisive for the prevention of OSCC development. Customarily, initiation of OSCC is drawn out by the frequent and direct exposure to multiple tobacco-derived carcinogens, and mostly by the nicotine present in tobacco products.

Nicotine is one of several natural alkaloids that can be readily absorbed by epithelial cell linings of buccal mucosa, respiratory tract, and via a basal layer of skin. However, the absorption and excretion of this alkaloid depend on the pH of the epithelial cell membrane.[6] Even though nicotine is considered a key compound for increasing cravings for various tobacco-derived products, it is not considered a carcinogen though it has been reported to induce angiogenesis and tumor growth acceleration.[7,8] Several reports have proved the dose-dependent primary effect of nicotine on the central nervous system along with the

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https://doi.org/10.1016/j.jobcr.2023.11.001
Received 24 May 2022; Received in revised form 3 October 2023; Accepted 6 November 2023
Available online 9 December 2023

effect on cell growth, attachment, and matrix protein synthesis.[9–12] Other reports have also studied the dose-dependent effect of nicotine on epithelial cells and demonstrated that the mitogenic effect of nicotine was associated with the increased risk of OSCC.[12]

A systemic review by Holiday et al. included many *in-vitro* studies that have investigated the effect of nicotine on human gingival, periodontal ligaments, and oral epithelial cells.[13] However, in that review only a single study performed on human oral epithelial cells was included which identified that the wide range of nicotine concentrations (100 nM - 1 mM; 24 h) failed to stimulate the production of interleukin-8.[14] Conversely several studies on human gingival fibroblasts (HGFs) or human periodontal ligament cells (HPDLCs) showed increased production of IL-8 and stimulated growth of human gingival epithelial cell in vitro with exposure to similar nicotine concentration (1 nM–1 mM; 24 h) [23,37,42,44,].[15–18] Unfortunately, the mentioned studies did not identify the effect of nicotine on cell proliferation and other possible modulations affecting the cell fate (Holiday et al., 2019).[13] Additionally, around 50 % of current and former cigarette smokers, including those who are probably developing potentially malignant oral dysplastic lesions, have started using a vaporized form of nicotine called e-cigarettes as a part of a smoking cessation intervention.[19] To this end, the determination of nicotine-induced malignant transformation in oral epithelial cells is extremely needed. Since many individuals with undiagnosed premalignant lesions have started using e-cigarettes in place of tobacco smoking, the aim of the present study was to understand the consequences of nicotine on oral epithelial cells via evaluating the nicotine-induced/mediated changes in cell proliferation, cell cycle, and PI3K/MAPK pathway activation on healthy human squamous epithelial cells.

2. Materials and methods

2.1. Cell culture conditions

Oral epithelial cells were obtained from the healthy buccal mucosa using a toothbrush and were washed with PBS. Cells were then washed with the antibiotic/antimycotic mixture twice before further processing. Cells were cultured with DMEM supplemented with 10 % fetal bovine serum, 2 % L-glutamine, and 1X antibiotic/antimycotic mixture in collagen-treated T25 cell culture flasks (SPL, Korea).

2.2. MTT assay

The effect of nicotine on cell proliferation was measured using MTT dye which gets reduced to purple-colored formazan crystals by mitochondrial dehydrogenase enzyme present in living cells. Briefly, 1×10^4 cells were seeded in a surface-treated polystyrene 96-well plate (Genetix Biotech, India) and were incubated overnight at 37 °C and 5 % CO_2 for adherence. On the next day, serially diluted nicotine was added to wells in triplicate at the concentration ranging from 5 mM to 300 mM and incubated at 37 °C and 5 % CO_2 for 48 h. After incubation, 10 μL of MTT (5 mg/mL) was added to each well, mixed well, and incubated for 3 h in the incubator. Reduced MTT was dissolved by adding 100 μL of DMSO to each well. Total reduced MTT was spectrophotometrically measured at 570 nm. A dose-response curve was utilized to determine the nicotine concentration required to proliferate the oral epithelial cells. All samples were kept in triplicate for result consistency.

2.3. Cell count and viability assay

Oral epithelial cells were treated with 150 mM nicotine hemisulphate salt solution (Sigma-Aldrich, MO) and incubated at 37 °C and 5 % CO_2 for 10 days with the passage every 2 days. Untreated cells were also cultured under the same conditions except for nicotine at the same time. The media along with 150 mM nicotine was replaced every third day and were passaged at 90 % confluence in both treated and untreated plates. After 10 days of incubation, treated and untreated cells were collected, washed with PBS, and were proceeded further for cell count and viability assay (Muse® Count &Viability Assay kit) as per the manufacturer's protocol using Muse cell analyzer (Muse® Cell Analyzer, Millipore, USA).

2.4. Cell cycle distribution assay

Cells treated with and without nicotine were incubated for 10 days in a T25 surface-treated flask. After 10 days of incubation, nicotine-treated, and untreated cells were synchronized for 24 h in serum-starved DMEM media and were then incubated with complete culture media for 24 h. After that, cells were harvested, washed twice with PBS, and fixed in cold 70 % ethanol overnight at −20 °C. Ethanol-fixed cells were pelleted down and were washed twice with PBS. Cells were then incubated with a cell cycle reagent (Muse™ Cell Cycle Kit) as per the manufacturer's protocol and were run on a Muse cell analyzer.

2.5. PI3K/MAPK pathway activation assay

To identify the mechanism responsible for the nicotine-induced proliferative activity we evaluated PI3K and MAPK pathway activation in nicotine treated cells using Muse PI3K/MAPK dual pathway activation kit (Muse™ PI3K/MAPK Dual Pathway Activation Kit) as manufacturer's protocol.

3. Results

3.1. Nicotine induces oral epithelial cell proliferation

An effect of nicotine on healthy oral epithelial cell proliferation was determined using an MTT assay. As described in materials and methods, when healthy oral epithelial cells were treated with the different concentrations of nicotine ranging from 5 mM to 300 mM, the proliferation of healthy oral epithelial cells was observed at 150 mM concentration of nicotine (Fig. 1). Exponentially growing cells were then treated with 150 mM concentration of nicotine for 10 consecutive days and were evaluated for cell count and viability assay. A significantly increased cell count was observed in the cells treated with nicotine as compared to control cells (Fig. 2). Overall, these data suggested that at 150 mM concentration nicotine induces the proliferation of healthy oral epithelial cells.

3.2. Nicotine drives cells in the first growth (G0/G1) phase

Nicotine-induced growth distribution was determined using a flow cytometry-based cell cycle distribution assay using a Muse cell analyzer. As shown in Fig. 3, in the control flask, 58.3 % of the cells were gated in S phase while, 36.0 % and 4.3 % of the cells were gated in G0/G1 and G2/M phases, respectively. On the other hand, cells after 10 days of treatment with nicotine showed a change in cell cycle distribution and showed an increased cell shift in the G0/G1 phase to 85.7 %. While the proportion of S phase cells was decreased to 4.6.0 %. However, profound changes in the G2/M phase were not noticeable.

3.3. Nicotine induces PI3K/MAPK dual pathway activation

To identify the probable responsible mechanism for increased cell proliferation, we evaluated PI3K and/or MAPK dual pathway activation after exposing cells to nicotine. Surprisingly, it was found that only 6.6 % of total nicotine unexposed cells failed to show activation of any of the oncogenic pathways despite presenting at the S phase. While 88 % of untreated cells demonstrated both the oncogenic pathway activation under usual culture conditions as shown in Fig. 4. However, after 10 days of continuous exposure to nicotine, 94.7 % of cells exhibited activation of PI3K/MAPK dual pathway activation despite their existence in

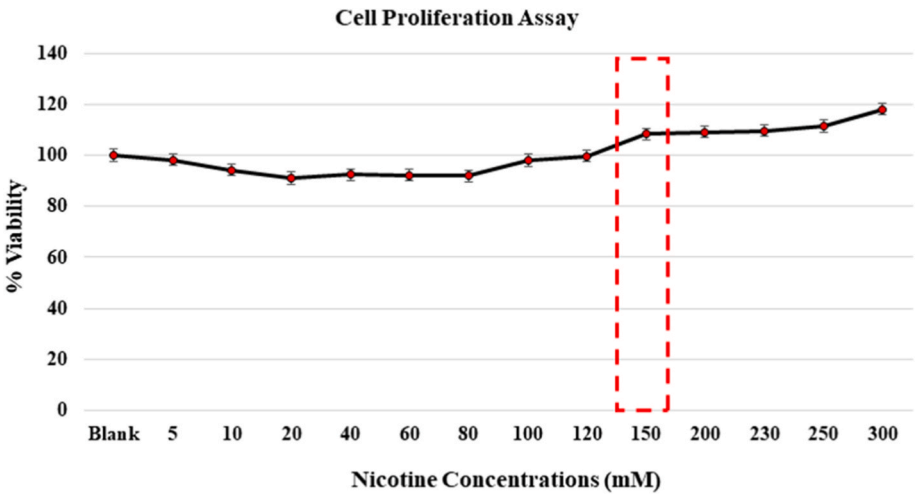

Fig. 1. Dose-response curve of healthy epithelial cells exposed to the different concentrations of nicotine.

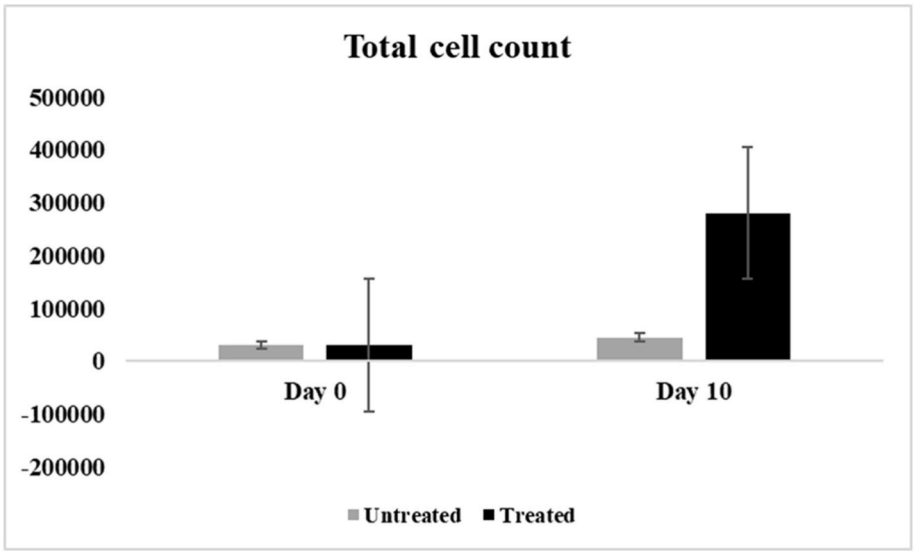

Fig. 2. Total cell count after 10 days of exposure with 150 mM nicotine.

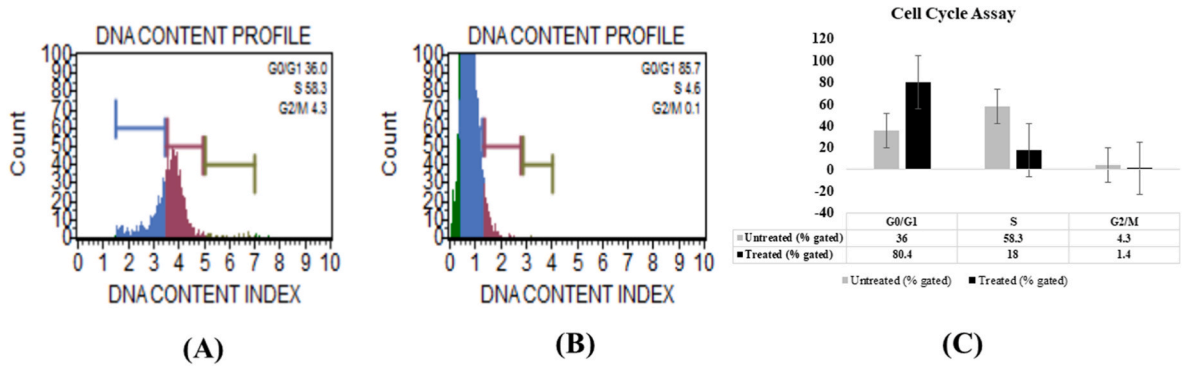

Fig. 3. Flow cytometry-based cell cycle distribution assay of (A) Control cells (B) Nicotine treated cells (C) Graphical representation of cell cycle distribution.

the G0/G1 phase (Fig. 4).

4. Discussion

Several case-control studies conducted in South Asia and cohort studies conducted in India and Pakistan have revealed that there is no safe form of tobacco and both combustible and smokeless tobacco are carcinogenic to humans.[20] Despite increasing knowledge of nicotine as a potent carcinogen present in various smokeless tobacco and other combustible tobacco products, very little is acknowledged concerning

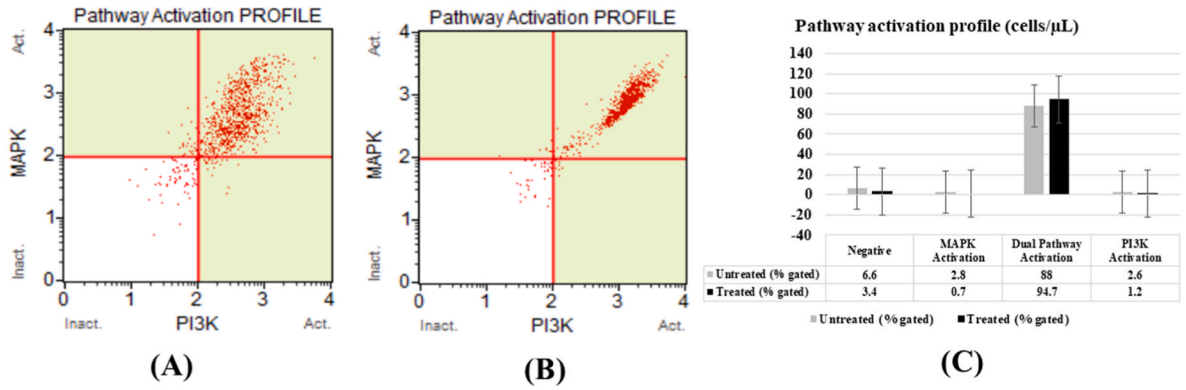

Fig. 4. Flow cytometry-based PI3K/MAPK dual pathway activation assay of (A) Control cells (B) Nicotine treated cells (C) Graphical representation of PI3K/MAPK dual pathway activation.

the modulating effect of nicotine on oral epithelial cells. However, some studies have identified that nicotine is not a carcinogen by itself, but it may have the potential to promote a migratory phenotype of a cell which is a cellular hallmark of malignancy.[6,21–25]

Several previous studies have demonstrated the effect of nicotine in premalignant oral keratinocytes, oral cancer cells, leukemias, and cervical and lung cancer cells[12,26–28] however, the effects of nicotine on healthy epithelial cells of the oral cavity is unknown which is a first encounter site with nicotine present in tobacco. Studies have reported that after a single exposure to smokeless tobacco (7.5 g) or a cigarette smoking in volunteers, plasma levels of nicotine reached a maximum of the single cigarette smoked.[20] The results of the present study demonstrated that the nicotine at the concentration of 150 mM initiates the proliferative activity of oral epithelial cells which was evaluated via the cell cycle and PI3K/MAPK dual pathway activation assays. PI3K/MAPK signaling pathways are key regulators of normal cellular processes such as cell growth, proliferation, and survival of the cells.[29,30] Hence, in the control cells activation of these pathway was observed in the 88 % of the cells presenting S phase of the cell cycle assay. While on the other hand, majority of the nicotine treated cells presented at the G0/G1 phase of the cell cycle indicating the cells are entering into the G0 phase very rapidly along with the PI3K/MAPK pathway activation (94.7 %) indicating dysregulated cell cycle regulatory or uncontrolled cell proliferative activity. Accordingly, in the previously reported study nicotine was found to enhance the expression of differentiation markers such as CK13, profilaggrin/filaggrin, and involucrin, in oral epithelial mucosal cells.[31] Additionally, Lee et al., 2005 published the same findings with the addition that nicotine has lethal effects on oral cancer cells at a dosage of 100 g/mL. In addition to that, our flow cytometry-based cell cycle assay, results demonstrated that at 150 mM concentration, cells were continuously divided and were found to be present in a first growth phase or post-mitotic phase.[32] However, several studies have reported the vulnerable cytotoxic effect of nicotine on premalignant and malignant cells.[31,32] The reason for this conflict could be attributed to the difference in the cellular behavior of primary keratinocytes and oral mucosal cells. Additionally, keratinocytes from other parts of the body and oral epithelial lining cells may respond to nicotine differently pharmacologically.[27] Furthermore, according to our research, Wisniewski et al., 2018 shown that nicotine has the capacity to increase the migratory phenotype of oral keratinocytes, which also aids in the development of OSCC.[6] Likely, other ligands and signaling pathways in crosstalk with the PI3K/Akt and MEK/ERK signaling pathways may affect their survival in an unknown fashion. Similarly, Nishioka et al., 2018 reported that the nicotine-treated oral cancer cells demonstrated increased proliferation mediated through activation of growth-related signaling pathways as compared to the control cells.[33]

To the best of our knowledge, this study provides the evidence that nicotine can induce healthy oral epithelial cells to proliferate by activating the PI3K and MAPK pathways, which is a key characteristic of cancer. Several studies have similarly reported that nicotine induces proliferation by activating the MEK/ERK pathway in cancers.[34–36] Since many current and former smokers have started using vaporized nicotine as a part of tobacco cessation intervention who may not have been diagnosed with any premalignant lesions, they are at increased risk to trigger the progression of OSCC.

Many of the *in-vitro* studies have investigated the range of nicotine at the dose of 1–10 µM to boost the proliferation.[37–39] In the current study, the lowest dose observed to boost the proliferation of epithelial cells mediated through PI3K/MAPK pathway activation was a 150 mM concentration of nicotine. However, studies exploring the nicotine content in smokeless tobacco and e-cig vapor vary. Hence, further studies demonstrating the potential exposed concentration of nicotine to the linings of the oral cavity are required to improve the understanding of the nicotine effect in the body. Although a form of nicotine used in the current study may not reproduce the cellular response to smokeless tobacco or vaporized nicotine derived from tobacco smoke, however, it helps in identifying the lowest dose of nicotine essential to induce malignant transformation along with its targeting pathways on oral epithelial linings. Collectively, the present study confirms the role of nicotine as an epithelial modulator possibly via dysregulating cell cycle checkpoints and activating the onco-signaling pathway.

There are certain limitations to current study. It is important to keep in mind that pathological processes are more complex than straightforward cell viability measurements. We excluded cotinine and kept the focus on nicotine. However, nicotine is converted in the body to cotinine which has a longer half-life than nicotine. Thus, in vivo, longer exposure to cotinine could cause cells to experience negative effects. Hence, inclusion of cotinine and accurate exposure concentration of nicotine in normal physiological conditions are important considerations for future studies.

5. Conclusion

The current study provides the first evidence that in squamous epithelial cells of the oral cavity, nicotine plays a crucial role in increasing cell proliferation. Our finding supports the concern about the consequence of nicotine exposure on cellular behavior which may act as a strong pro-oncogenic signaling promoter to activate the malignant transformation through activating oncogenic pathways.

Funding

This research did not receive any specific funding from funding agencies in the public, commercial and not-for-profit sectors.

Declaration of competing interest

There is no conflict of interest by any of the authors to declare.

Acknowledgment

Authors acknowledge sincerely the facility provided by The Gujarat Cancer & Research Institute, Ahmedabad, India.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca - Cancer J Clin*. 2021;71:209–249.
2. Ferlay J, Colombet M, Soerjomataram I, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer*. 2019;144:1941–1953.
3. Wisniewski DJ, Ma T, Schneider A. Advances in the chemopreventive targeting of oral carcinogenesis. *Current Oral Health Rep*. 2015;2:63–72.
4. Amagasa T, Yamashiro M, Uzawa N. Oral premalignant lesions: from a clinical perspective. *Int J Clin Oncol*. 2011;16:5–14.
5. Global Burden of Disease Cancer C, Fitzmaurice C, Allen C, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the global burden of disease study. *JAMA Oncol*. 2017;3:524–548.
6. Wisniewski DJ, Ma T, Schneider A. Nicotine induces oral dysplastic keratinocyte migration via fatty acid synthase-dependent epidermal growth factor receptor activation. *Exp Cell Res*. 2018;370:343–352.
7. Drope J, Cahn Z, Kennedy R, et al. Key issues surrounding the health impacts of electronic nicotine delivery systems (ENDS) and other sources of nicotine. *Ca - Cancer J Clin*. 2017;67:449–471.
8. Heeschen C, Jang JJ, Weis M, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat Med*. 2001;7:833–839.
9. Nair MP, Kronfol ZA, Schwartz SA. Effects of alcohol and nicotine on cytotoxic functions of human lymphocytes. *Clin Immunol Immunopathol*. 1990;54:395–409.
10. Hanes PJ, Schuster GS, Lubas S. Binding, uptake, and release of nicotine by human gingival fibroblasts. *J Periodontol*. 1991;62:147–152.
11. Boutherin-Falson O, Blaes N. Nicotine increases basal prostacyclin production and DNA synthesis of human endothelial cells in primary cultures. *Nouv Rev Fr Hematol*. 1990;32:253–258.
12. Waggoner SE, Wang X. Effect of nicotine on proliferation of normal, malignant, and human papillomavirus-transformed human cervical cells. *Gynecol Oncol*. 1994;55:91–95.
13. Holliday RS, Campbell J, Preshaw PM. Effect of nicotine on human gingival, periodontal ligament and oral epithelial cells. A systematic review of the literature. *J Dent*. 2019;86:81–88.
14. Desjardins J, Grenier D. Neutralizing effect of green tea epigallocatechin-3-gallate on nicotine-induced toxicity and chemokine (C-C motif) ligand 5 secretion in human oral epithelial cells and fibroblasts. *J Investig Clin Dent*. 2012;3:189–197.
15. Wendell KJ, Stein SH. Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. *J Periodontol*. 2001;72:1038–1044.
16. Kashiwagi Y, Yanagita M, Kojima Y, Shimabukuro Y, Murakami S. Nicotine up-regulates IL-8 expression in human gingival epithelial cells following stimulation with IL-1beta or P. gingivalis lipopolysaccharide via nicotinic acetylcholine receptor signalling. *Arch Oral Biol*. 2012;57:483–490.
17. Mahanonda R, Sa-Ard-Iam N, Eksomtramate M, et al. Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res*. 2009;44:557–564.
18. Wu L, Zhou Y, Zhou Z, et al. Nicotine induces the production of IL-1beta and IL-8 via the alpha7 nAChR/NF-kappaB pathway in human periodontal ligament cells: an in vitro study. *Cell Physiol Biochem*. 2014;34:423–431.
19. Schoenborn CA, Gindi RM. *Electronic Cigarette Use Among Adults: United States, 2014*. NCHS Data Brief; 2015:1–8.
20. Warnakulasuriya S, Straif K. Carcinogenicity of smokeless tobacco: evidence from studies in humans & experimental animals. *Indian J Med Res*. 2018;148:681–686.
21. Woo S, Gao H, Henderson D, et al. AKR1C1 as a biomarker for differentiating the biological effects of combustible from non-combustible tobacco products. *Genes*. 2017;8.
22. Macha MA, Matta A, Chauhan SS, Siu KW, Ralhan R. Guggulsterone (GS) inhibits smokeless tobacco and nicotine-induced NF-kappaB and STAT3 pathways in head and neck cancer cells. *Carcinogenesis*. 2011;32:368–380.
23. Clark CA, McEachern MD, Shah SH, et al. Curcumin inhibits carcinogen and nicotine-induced Mammalian target of rapamycin pathway activation in head and neck squamous cell carcinoma. *Cancer Prev Res*. 2010;3:1586–1595.
24. Niu W, Zhang M, Chen H, et al. Peroxiredoxin 1 promotes invasion and migration by regulating epithelial-to-mesenchymal transition during oral carcinogenesis. *Oncotarget*. 2016;7:47042–47051.
25. Yu CC, Chang YC. Enhancement of cancer stem-like and epithelial-mesenchymal transdifferentiation property in oral epithelial cells with long-term nicotine exposure: reversal by targeting SNAIL. *Toxicol Appl Pharmacol*. 2013;266:459–469.
26. Konno S, Chiao JW, Wu JM. Effects of nicotine on cellular proliferation, cell cycle phase distribution, and macromolecular synthesis in human promyelocytic HL-60 leukemia cells. *Cancer Lett*. 1986;33:91–97.
27. Maneckjee R, Minna JD. Opioid and nicotine receptors affect growth regulation of human lung cancer cell lines. *Proc Natl Acad Sci U S A*. 1990;87:3294–3298.
28. Schuller HM. Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochem Pharmacol*. 1989;38:3439–3442.
29. Rascio F, Spadaccino F, Rocchetti MT, et al. The pathogenic role of PI3K/AKT pathway in cancer onset and drug resistance: an updated review. *Cancers*. 2021;13.
30. Yuan J, Dong X, Yap J, Hu J. The MAPK and AMPK signalings: interplay and implication in targeted cancer therapy. *J Hematol Oncol*. 2020;13:113.
31. Kwon OS, Chung JH, Cho KH, et al. Nicotine-enhanced epithelial differentiation in reconstructed human oral mucosa in vitro. *Skin Pharmacol Appl Skin Physiol*. 1999;12:227–234.
32. Lee HJ, Guo HY, Lee SK, et al. Effects of nicotine on proliferation, cell cycle, and differentiation in immortalized and malignant oral keratinocytes. *J Oral Pathol Med*. 2005;34:436–443.
33. Nishioka T, Tada H, Ibaragi S, Chen C, Sasano T. Nicotine exposure induces the proliferation of oral cancer cells through the alpha7 subunit of the nicotinic acetylcholine receptor. *Biochem Biophys Res Commun*. 2019;509:514–520.
34. Dasgupta P, Rastogi S, Pillai S, et al. Nicotine induces cell proliferation by beta-arrestin-mediated activation of Src and Rb-Raf-1 pathways. *J Clin Invest*. 2006;116:2208–2217.
35. Kamer AR, El-Ghorab N, Marzec N, Margarone 3rd JE, Dziak R. Nicotine induced proliferation and cytokine release in osteoblastic cells. *Int J Mol Med*. 2006;17:121–127.
36. Khorram O, Han G, Magee T. Cigarette smoke inhibits endometrial epithelial cell proliferation through a nitric oxide-mediated pathway. *Fertil Steril*. 2010;93:257–263.
37. Calderon LE, Liu S, Arnold N, Breakall B, Rollins J, Ndinguri M. Bromoenol lactone attenuates nicotine-induced breast cancer cell proliferation and migration. *PLoS One*. 2015;10, e0143277.
38. Sun H, Ma X. alpha5-nAChR modulates nicotine-induced cell migration and invasion in A549 lung cancer cells. *Exp Toxicol Pathol*. 2015;67:477–482.
39. Yuge K, Kikuchi E, Hagiwara M, et al. Nicotine induces tumor growth and chemoresistance through activation of the PI3K/Akt/mTOR pathway in bladder cancer. *Mol Cancer Therapeut*. 2015;14:2112–2120.