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ORIGINAL ARTICLE

Vitamin C stimulates RNA expression of human gingival fibroblasts proliferation and adhesion in cigarette smokers: An *in vitro* study

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KEYWORDS

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Abstract *Background:* Smoking and the severity of periodontal disease have long been associated. In Saudi Arabia, tobacco smoking is rising, contributing to the increased demand for products that counter its detrimental effects. The antioxidant properties of vitamin C (vit C) make it a powerful countermeasure to tobacco toxicity. Observation of these effects on human gingival fibroblasts (hGFs) would suggest use of vitamin C in future dental applications.

Aim: To examine the proliferation, adhesion, and expression of extracellular RNA in human gingival fibroblasts extracted from cigarette smokers when compared to never-smokers, in association with vitamin C.

Materials and Methods: Human gingival fibroblasts were extracted from Periodontal free sites of healthy adult male participants. Group 1; consisted of Heavy cigarette smokers (n = 1) while group 2 was never-smokers (n = 1). Collected cells were cultured and subcultured in supplemented growth medium. Vitamin C was then induced in the medium at the experimental sixth passage. RNA expression analysis using quantitative reverse transcriptase-polymerase chain reaction was performed to analyze the adhesion, proliferation, and extracellular matrix expression.

Results: Expression of the adhesion gene (*CD44*) in the smoker group was significantly downregulated than never-smoker group (p-value = 0.024). After the induction of vitamin C, the smoker samples showed a significant improvement in their gene expression levels. The extracellular genes involved in this study (*COL1A1*, *LAMA3*, and *TGFB3*) were significantly affected by the smoking

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status. In addition, the proliferation of *MKI67* and *CCNB1* genes in smokers and never-smokers was increased.

Conclusion: Cigarette smoking affects the overall properties of human gingival fibroblasts' adhesion, proliferation, and extra-cellular matrix formation. Furthermore, the addition of vitamin C affects these cellular properties in a positive manner.

1. Introduction

The dental supporting unit, known as the periodontium, is a complex organ composed of four components of mesenchymal tissue (gingiva, cementum, alveolar bone, and periodontal ligament). These components provide the tooth with an attachment apparatus to the jawbone that is capable of withstanding masticatory forces, maintaining homeostasis within the oral cavity, and protecting against oral pathogens (Melcher, 1976). Fibroblasts are ubiquitous cellular constituents of masticatory gingival connective tissues. In culture, they can be distinguished by their morphological characteristics, including spindle-shaped appearance, adherent growth on plastic, location within the body, and lack of distinctive lineage markers (Sahai et al., 2020). Moreover, human gingival fibroblasts (hGFs) play a crucial role in the secretion and degradation of the extracellular collagen matrix, which provides structural integrity and participates in wound healing and tissue regeneration, immunological regulation, mechanotransduction, angiogenesis, and organ fibrosis (Kabakov et al., 2021). To examine how fibroblasts are heterogeneous and adapt to their original tissues, fibroblasts extracted from masticatory gingival tissue were compared to oral lining mucosa in their genetic composition and proliferation (Kabakov et al., 2021). A significant difference was noted in the expression of extra-cellular matrix (ECM) collagens and enzymes (*COL1A1*, *COL1A2*, *COL3A1*, *COL4A5*, *COL4A6*, *COL5A1*, and *COL5A2*) in fibroblasts from the lining mucosa compared with gingival fibroblasts (Kabakov et al., 2021). Researchers have interpreted these results as a characteristic feature that distinguishes different oral fibroblasts and suggested that this might be a consequence of the high turnover rate of the epithelium underneath the non-keratinized oral lining. However, hGFs exhibited a higher proliferation rate of 10–30%, as measured by the expression of common gene markers (*MKI67*, *PCNA*, *MCM2-6*, *MYBL2*, *BUB1*, *PLK1*, *CCNE1*, and *CCNB1*). Further discussion concerns the transforming growth factor-β (*TGF-β*) signaling molecules, which play an integral role in the enhanced production of ECM and the physiological wound healing process and may be associated with gingival fibrosis and excessive scarring. These molecules are abundant in masticatory gingival fibroblasts (Kabakov et al., 2021). In addition, gingival fibroblast possesses a distinctive phenotype that respond to various extrinsic factors by taking on several alternative destinations. This is evident from their behavioral adaptation in those who smoke. Tobacco smoking dramatically increases the risk of developing several pathological conditions that lead to unfortunate deaths (Tonetti, 1998). In Saudi Arabia, tobacco smoking prevalence represents as 17.9% out of 12,800 sampled households in 2019, according to the Global Adult Tobacco Survey (GATS) (Health, 2019). Smoking is one of the most difficult habits because of the addictive properties of nicotine. Furthermore, the body is also exposed to over 4,000 toxic substances in cigarettes that generate free radicals, thereby damaging DNA, leading to cancerous cell formation (Geiss and Kotzias, 2007, Kovacs et al., 2012). Compared with never-smokers, in smokers, the combustion of tobacco smoke causes significantly deteriorated periodontal health parameters, including; higher rates of attachment loss, missing teeth, gingival recession, and inferior post treatment response (Albandar et al., 2000). A relationship between smoking and periodontal disease has been established and investigated (Grossi et al., 1994, Periodontology, 1996). There have been a series of discussions regarding this relationship, culminating in a consensus report published in 2018 on a revised Classification of Periodontal and Peri-Implant Diseases and Conditions that recognizes tobacco smoking as a risk factor for periodontal disease progression and for analyzing the likelihood of successful treatment (Papapanou et al., 2018, Tonetti et al., 2018). Most of the adverse effects of tobacco products on periodontal tissues can be attributed to direct inhibition of normal fibroblast function (Lallier et al., 2017). Human gingival fibroblasts (hGFs) metabolize and accumulate significant quantities of nicotine, with most remaining inside the fibroblasts, where it can interfere with cell metabolism or other functions. In addition, unmetabolized nicotine can be released into the environment. Thus, several cellular processes may be disrupted, resulting in cytotoxicity (Hanes et al., 1991, Wyganowska-Swiatkowska and Nohawica, 2015). Under the influence of nicotine exposure, the attachment and adhesion strengths of hGFs to glass and human root surfaces were investigated. Nicotine appears to inhibit the attachment of hGFs, jeopardizing the healing process after periodontal therapy (Tanur et al., 2000). Vitamin C (vit C), also known as ascorbic acid, is essential for the proper folding and deposition of collagen proteins and plays a significant role in maintaining ECM homeostasis as well as being a potent antioxidant. Furthermore, vit C has been identified as a critical regulator of stem cell identity and behavior that influences pluripotency, self-renewal, and differentiation by enhancing cell reprogramming. vit C cannot be synthesized in the human body; therefore, it must be constantly replenished by the diet (D'Aniello et al., 2017). Accordingly, recent studies have suggested that supplementation with vitamin C may be beneficial in alleviating the symptoms of the common cold and COVID-19 (Ran et al., 2018, Hemilä et al., 2021). In addition, vit C counteracts the harmful effects of tobacco (Falsafi et al., 2016). Supplementing cigarette-effected hGFs with vit C systemically, locally, or in a combination showed positive effects on their morphology, viability, proliferation, migration, and apoptosis (Torshabi et al., 2017). To the best of our knowledge, few studies has been

performed on gingival fibroblasts isolated from smokers, rather than simply exposing healthy gingival fibroblasts to a few, but not all of the chemicals present in cigarettes (Lallier et al., 2017, Torshabi et al., 2017, Tatsumi et al., 2021). This study aimed to examine the proliferation, adhesion, and expression of extracellular RNA in human gingival fibroblasts extracted from cigarette smokers when compared to never-smokers in association with vitamin C.

2. Materials and methods

2.1. Ethical guidelines

This study was conducted in accordance with the standards and protocols of King Saud University (KSU) and was approved by the Ethical Committee of the Institutional Review Board at KSU (Project No. E-18-3071) and College of Dentistry Research Center (CDRC No. PR0077). Informed consent was obtained from all the participants. The work protocol of the study was conducted using the facility and support of the Stem Cell Unit Laboratory at the Department of Anatomy, College of Medicine, KSU, Riyadh, Saudi Arabia.

2.2. Human gingival tissue explantation

Healthy gingival connective tissue was collected from two systemically healthy adult male donors aged $\geq$ 20 years. Inclusion criteria included clinically healthy gingiva without signs of inflammation (erythematous, edematous, or bleeding). While exclusion criteria was known systemic diseases or treated with antibiotics in the past month. Gingival samples were obtained following dental surgical procedures that required excision of gingival tissues, such as a third molar extraction procedure, gingvectormies or periodontal crown lengthening surgery at the College of Dentistry, KSU in Riyadh, Saudi Arabia. Furthermore, human participants were divided into two main groups. The first group (N-N) consisted of never (cigarette, hookah, or vapor) smokers, whereas the second group represented heavy cigarette users who were defined as smoking $\geq$ 20 cigarettes/day for $\geq$ 2 years and were known as (S-N) (Kaldahl et al., 1996). The gingival tissue samples were treated aseptically and transferred into 15 mL centrifuge tubes (BD Falcon™, NJ, USA) containing fibroblast culture media to the laboratory for direct explant culture under a laminar flow hood.

2.3. Culturing of hGFs

The excised tissues were washed three times using Phosphate Buffered saline (PBS; Gibco® Life Technologies, Roskilde, Denmark) supplemented with 10,000 IU/mL Penicillin-Streptomycin (Pen-Strep; Gibco®, Carlsbad, CA, USA). Specimens were minced into approximately 2–3 mm pieces and placed in the center of each well of six-well tissue culture plates (Corning®, Tewksbury, MA, USA). Thereafter, explants were covered with a minimal amount of fibroblast culturing media (~0.5 mL) containing Dulbecco's modified Eagle's medium-high glucose (DMEM; Gibco®, BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®, Grand Island, NY, USA), 1% Pen-Strep (Gibco®, Carlsbad, CA, USA), and 1% MEM non-essential amino acids (Invitrogen Gibco®, Grand Island, NY, USA) to facilitate the attachment of tissues to the culture plate surface. Tissues were incubated at 37 °C for 30 min in a humidified atmosphere of 5% CO_2 and 95% oxygen before adding 1 mL of a new medium. Every 72 h, the medium was replaced with fresh medium until the migration of cells from the tissue explant was evident.

2.4. Subculture and expansion of hGFs

Furthermore, non-adherent cells were removed, and fresh media was added until migrated cells reached a confluency of 80–90%. A phase-contrast inverted microscope (Carl Zeiss®, Axio Observer A1 inverted microscope, Leica, Berlin, Germany) was used to determine whether cells had reached the desired confluence. The plastic-adherent confluent cells were passaged with 0.25% trypsin/EDTA (Gibco®, Carlsbad, CA, USA) for 2–3 min at 37 °C with gently rocking of flasks intermittently. Trypsin neutralization was achieved using complete DMEM (twice the volume of the trypsin/EDTA reagent). The cell suspensions were then centrifuged at 1,500 rpm for 5 min. Thereafter, supernatants were aspirated and discarded. The cell pellets were then resuspended in 5 mL of complete medium to increase the cell numbers. After diluting the cell pellet and dividing it into two T-75 flasks, a fresh growth medium was added to expand the cells. The cells were routinely observed using an inverted phase-contrast microscope. Experiments were conducted using cells from the sixth passage.

2.5. RNA expression analysis

Cells were seeded in a six-well plate (Falcon™ Polystyrene Microplates 6-well; non-treated; flat-bottom; growth area: 9.6 cm^2; well volume: 15.5 mL). Vitamin C, also known as ascorbic acid, at a concentration of 200 μM, was prepared as a solution by diluting 14.45 g ascorbic acid 2-phosphate in sterile 1 L H_2O to obtain a 50 mM solution, filtered, stored at 4 °C, and then this solution was added to the culture medium as described previously (Van Pham et al., 2016). Vitamin C solution was then added based on the group assembly: (1) Never smoke fibroblasts without vit C addition (N-N Group), (2) Never smoke fibroblasts with vit C addition (N-C Group), (3) Smoker fibroblasts without vit C addition (S-N Group), and (4) Smoker fibroblasts with vit C addition (S-C Group). Total RNA was isolated after 3 days of incubation, following the manufacturer's instructions for the RNAase extraction kit (Analytik Jena AG, Thuringia, Germany). After removing the residual medium, the lysis solution from the kit was added at 133 μL/well of 6 wells culture plate and gently washed with cold, sterile PBS. The lysed cells were incubated for 2–3 min at room temperature, and the final yield of total RNA was obtained. The complete disruption and lysis of the cells was achieved with the aid of tissue scrubber, which was used to scrub the base of the wells gently. The lysed cells were collected in an Eppendorf microcentrifuge tube and preserved at −80 °C until sample collection. RNA extraction was performed using the RNAase extraction kit (Analytik Jena AG). The concentration of total RNA was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

Complementary DNA (cDNA) was synthesized using the Thermo Fisher Scientific High-Capacity cDNA transcription

kit and the ProFlex PCR system. Gene expression levels were determined using real-time PCR (Thermo Fisher Scientific, VIA 7 system) with a qRT-PCR kit (Applied Biosystems, Warrington, UK). PCR reactions were performed using the Fast SYBR Green PCR Master to amplify the purchased primers as predesigned products (Takara Bio Inc., Otsu, Japan), as shown in Table 1. All reactions were performed in triplicates. All values were normalized to the expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the qRT-PCR products were quantified using the previously described 2-ΔΔCt method (Spicer et al., 2012). The panel of genes studied included collagen type I alpha 1 chain (*COL1A*), laminin subunit alpha 3 (*LAMA3*), transforming growth factor (*TGF-B3* and *TGFR2*), CD44 antigen (*CD44*), cyclin B1 (*CCNB1*), marker of proliferation Ki-67 (*MK167*); The sequences of genes are shown in Tables 1.

3. Statistical analysis

The experiment was replicated three times for each gene. Data are presented as mean, median, and standard deviation. Statistical analyses were conducted using IBM SPSS version 21 (IBM, New York, NY, USA). At a significance level of 0.05, the Kruskal–Wallis test and post-hoc test were used to compare the results among the groups. In addition, we conducted another pairwise comparison test using both the Mann–Whitney *U* test and Wilcoxon signed-rank test to compare the mean ranks of human gingival fibroblasts between smokers' status and further comparison before and after Vit. C treatment.

4. Results

Table 2 presents the mean standard deviation and median interquartile range of various genes according to their main cellular functions: adhesion, proliferation, extracellular matrix formation.

In the cellular adhesion expression, the mean levels of *CD44* expression of human gingival fibroblasts differed statistically between the study groups (p-value = 0.024; Table 3). After performing a post hoc analysis, it was found that smokers without vit C induction had significantly lower expression in human gingival fibroblasts (S-N 2.00, $p < 0.05$) compared with the subjects from other three groups, S-C, N-N, and N-C (5.00, 10.00, 11.00, respectively, $p < 0.05$). Following the induction of vit C, the smoker samples showed significant improvement in gene expression levels (S-C 10.00, $p < 0.05$). The Mann–Whitney *U* test statistic value and mean ranks of smokers and neve-smokers (6.00, 2.00, and 5.00, respectively) showed significantly low values for smokers.

Regarding the extracellular genes involved in this study, collagen type-1 was strongly expressed in non-vitamin C-induced never-smoker samples (N-N 9.67, $p < 0.05$). Furthermore, it was notable that *LAMA3* was considerably higher in smokers S-N and S-C than in never-smokers (N-N and N-C). In contrast, the expression of isomere-3 of the tissue growth factor beta-3 (*TGFB3*) was significantly lower in smokers before and after vit C induction (S-N = 3.33; S-C = 3.67; $p < 0.05$) than never-smokers (N-N = 8.67; N-C = 10.33; $p < 0.05$). Furthermore, the *TGFBR2* level in the N-C group was significantly higher (11.00, $p < 0.05$) than that in the remaining three groups. As shown in Table 3, the Kruskal–Wallis test was used to compare the means of individual gingival fibroblast samples in terms of specific target genes.

There were no significant differences between the groups regarding the expression of *MK167*. *CCNB1* expression was lower in smokers S-N and S-C, with the highest level being reached at the end of vit C induction in the never-smoker group N-C 11.00, $p < 0.05$ (Fig. 1). According to the Wilcoxon signed-rank test statistic value for the never-smokers group before and after vit C induction, there was a significant increase in proliferative activity after the induction of vit C (Wilcoxon signed-rank test statistic value = -2.201, p-value = 0.028).

Table 1 List of targeted primers [Sequence (5′–3′)].

Cellular Function	Name	Sequence (5′–3′)
Extra-cellular markers	**COL1A - FP**	CCCGGGTTTCAGAGACAACTTC
	COL1A - RP	TCCACATGCTTTATTCCAGCAATC
	LAMA3 - FP	GTTCCAGAGCTGAGAGGCCAC
	LAMA3 - RP	GAGGCCCTCACCTGGATGGT
	TGFBR-2 - FP	GGAACTCCTGAGTGGTGTGGGAG
	TGFBR-2 - RP	GACCCCGCTGCTCGTCATAGA
	TGF-B3 - FP	GGGAGTTGCGCTAAGCAAT
	TGF-B3 - RP	CTCACCTCTCCCTCGATCATATTTC
Adhesion markers	**CD44 - FP**	CAGCACCATTTCAACCACAC
		CATTTCTGTCTACATCAGTCATCC
	CD44 - RP	
Proliferation markers	**MK167 - FP**	GAGGTGTGCAGAAAATCCAAA
	MK167 - RP	CTGTCCCTATGACTTCTGGTTGT
	CCNB1 - FP	CCTCCGGTGTTCTGCTTC
	CCNB1 - RP	TTCAGCATTAATTTTCGAGTTCC
Reference marker	**GAPDH-FP**	TGA AGG TCG GAG TCA ACG GAT
	GAPDH-RP	TCA CAC CCA TGA CGA ACA TGG

FP: forward primer; RP: reverse primer.
Collagen type I alpha 1 chain (*COL1A*), laminin subunit alpha 3 (*LAMA3*), transforming growth factor (*TGF-B3* and *TGFR2*), CD44 antigen (*CD44*), cyclin B1 (*CCNB1*), marker of proliferation Ki-67 (*MK167*).

Table 2 Descriptive statistics of human gingival fibroblasts in relation to the target genes.

Target genes	Study groups*—Mean (Standard deviation)				Study groups*—Median (Inter quartile range)			
	S-N	**S-C**	**N-N**	**N-C**	**S-N**	**S-C**	**N-N**	**N-C**
COLIA	1.006 (0.13)	0.402 (0.05)	1.004 (0.11)	0.824 (0.06)	1.061 (NA)	0.373 (NA)	1.024 (NA)	0.856 (NA)
LAMA3	0.009 (0.0004)	0.009 (0.00002)	0.002 (0.002)	0.003 (0.0001)	0.009 (NA)	0.009 (NA)	0.003 (NA)	0.003 (NA)
TGFBR2	1.000 (0.02)	0.700 (0.02)	1.000 (0.037)	1.138 (0.08)	1.001 (NA)	0.705 (NA)	0.996 (NA)	1.117 (NA)
TGFB3	0.549 (0.007)	0.550 (0.01)	0.897 (0.09)	0.964 (0.04)	0.548 (NA)	0.550 (NA)	0.853 (NA)	0.941 (NA)
CD44	0.114 (0.005)	0.158 (0.011)	1.037 (1.46)	0.203 (0.009)	0.116 (NA)	0.155 (NA)	0.194 (NA)	0.198 (NA)
MK167	1.00 (0.23)	1.065 (0.07)	1.546 (1.17)	2.597 (0.054)	0.998 (NA)	1.023 (NA)	2.13 (NA)	2.61 (NA)
CCNB1	2.143 (0.08)	2.151 (2.08)	8.629 (0.082)	10.92 (0.342)	2.104 (NA)	2.267 (NA)	8.602 (NA)	10.80 (NA)

Table 3 Comparison of mean rank values of human gingival fibroblast samples among individual targeted genes.

Target genes	Study groups*—Mean ranks				Kruskal-Wallis -test statistic	p-value
	S-N	**S-C**	**N-N**	**N-C**		
COLIA	8.67	2.00	9.67	5.67	8.231	0.041*
LAMA3	10.00	9.00	3.00	4.00	8.538	0.036*
TGFBR2	6.67	2.00	6.33	11.00	9.359	0.025*
TGFB3	3.33	3.67	8.67	10.33	8.641	0.034*
MK167	3.67	5.33	6.00	11.00	6.897	0.075
CCNB1	3.00	4.00	8.00	11.00	9.462	0.024*
CD44	2.00	5.00	9.00	10.00	9.462	0.024*

S-N = Smoker without mediation; S-C = Smoker with Vitamin C; N-N = Never smoker without medication; N-C = Never smoker with Vitamin C.
* Significant p-value ($P \leq 0.05$).

5. Discussion

Fibroblasts in the human body are particularly sensitive to the surrounding matrix and its components, growth factors, and cytokines and respond accordingly. The cellular environment can be altered by smoking tobacco. There has been long-standing research into the ability of nicotine to promote matrix degrading activity in gingival fibroblasts *in vitro*, as well as impairing its functions in preserving or repairing gingival connective tissue structures when healthy or diseased (Tipton and Dabbous, 1995). In an *in vitro* study, Tanur et al. observed that cigarette smoke condensate altered root surface cell attachment, resulting in significant changes in biological characteristics, such as cell viability and migration (Silva et al., 2012). In addition, a systematic review of the literature regarding the effect of tobacco smoking on human gingival fibroblasts suggests that tobacco smoking modifies the proliferation and adhesion of these cells to root surfaces. This process may also be cytotoxic due to tobacco components, which may explain the increase in the severity and incidence of periodontal disease in chronic tobacco users (Wyganowska-Swiatkowska and Nohawica, 2015). Vitamin C plays several important physiological roles in the body, including healing tissue damage by participating in hydroxylation reactions required for collagen synthesis. The correlation between periodontal disease and the micronutrient vit C was revealed by sailors with putrid gums due to their diet lacking vitamin C. Lack of vit C in the body results in scurvy, which is characterized by defective collagen production by fibroblasts (Sutton, 2003). Various forms of systemic vitamin C administration have been demonstrated to counteract the effects of cigarette smoking, including mouthwash, paste, and tablets (Møller et al., 2004, Falsafi et al., 2016). In contrast, it may be used as a complementary treatment approach to nonsurgical methods for persistent gingival inflammation via local injection (Yussif et al., 2016). The mechanism by which the direct local administration of vit C induces alterations in gingival fibroblast behavior and accelerates the positive outcome remains unclear. This study examined the expression of human gingival fibroblast RNA in relation to extracellular signaling, proliferation, and adhesion, even after drug administration of vit C to cigarette smokers *in vitro*. Using human gingival fibroblasts as a model, which were isolated from gingival connective tissue treated with medium containing vit C in both healthy never-smokers and heavy cigarette smokers. Several studies have explored the effects of

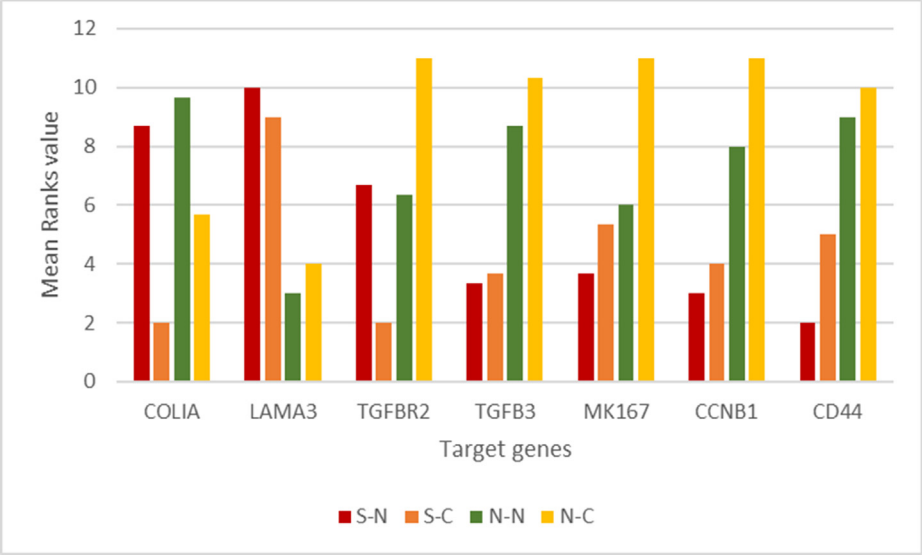

Fig. 1 Mean Ranks of human gingival fibroblast in target gene expression.

nicotine, either as an isolated compound or as a soluble cigarette smoke condensate (CSC) on cells (Torshabi et al., 2017). In addition, some researchers have developed devices that vaporize cells inside a closed chamber to simulate cigarette smoke (Semlali et al., 2011). In all the experiments, the ultimate goal was to promote the optimal function of approximately 4,000 toxic compounds found in cigarettes inhaled by the human body (Geiss and Kotzias, 2007). To overcome this issue, in this study, we used cells that had been exposed to cigarette toxics for years and had been genetically and structurally modified to cope with and thrive in such an environment. Fibroblasts are cells that are repeatedly exposed to cigarette vapors and absorb the toxins contained in them (Hanes et al., 1991). Furthermore, they are involved in the orchestration of wound healing, which involves various signaling cascades involving cytokines, chemokines, and growth factors. These cascades propagate extracellular matrix and collagen synthesis as well as promote proliferation and adhesion (García et al., 2020). As demonstrated by our RT-PCR experiments, collagen type I (*COL1A1*) was highly expressed in healthy individuals who did not smoke, indicating normal fibroblast function. Even with induced vitamin C, the gene was not significantly upregulated compared to control hGFs, regardless of the smoking condition. In contrast to the results obtained by Chaitrakoonthong et al., rinsing hGFs with vit C led to a significant increase in *COL1* gene expression (Chaitrakoonthong et al., 2020). Because their methodology does not coincide with ours, the comparison is inaccurate. Healthy hGFs cells were rinsed with different concentrations of vit C for certain periods of time, while we used an established concentration of vit C as instructed by Van Pham et al. (Van Pham et al., 2016, Chaitrakoonthong et al., 2020). The results of Tsutsumi et al. were in agreement with our results, which showed that L-ascorbic acid did not induce the expression of collagen mRNA. According to their hypothesis, a single treatment with normal L-ascorbic acid *in vitro* will have no collagen effect in comparison to daily use *in vivo* that persists in cells at high concentrations. Although L-ascorbic acid 2-phosphate magnesium salt is a long-lasting L-ascorbic acid derivative developed to improve the stability of vitamin C, it has been found to be more effective in expressing the collagen I gene in human fibroblasts than non-modified vit C (Tsutsumi et al., 2012). Furthermore, one of the major components of the basement membrane is the glycoprotein laminin (*LAMA3*), which was significantly more abundant in smokers before and after vit C stimulation than in never-smokers. However, both smokers and never-smokers experienced an increase in *TGFB3* following the administration of vitamin C. Chaitrakoonthong et al. revealed that vitamin C generally induces the production of extracellular matrix proteins and collagen, which is in agreement with our general data regarding ECM-targeted genes (Chaitrakoonthong et al., 2020).

The proliferation markers *MK167* and *CCNB1* were used to analyze gingival fibroblast proliferation in this study. Compared to those in never-smokers, both genes were expressed less in smokers; however, induction of vit C on hGFs increased their expression. Similarly, the expression of the *CD44* adhesion marker on hGFs increased after vit C stimulation. Vermehren et al. confirmed that smoking has an adverse effect on human gingival fibroblasts via a significant reduction in proliferation 24 h after cigarette exposure (Vermehren et al., 2020). Studies have found that minimum exposure to nicotine can inhibit the attachment of cells, whereas high concentrations are required to inhibit cell proliferation. However, the studies frequently reported contradictory results as a result of inconsistent methodology, which makes it difficult to draw any firm conclusions (Holliday et al., 2019). A recent study supports the hypothesis that when hGFs are exposed to nicotine or CSCs, their proliferation and migration are greatly inhibited. This impairs wound healing, triggers an inflammatory response, and contributes to the development of clinical manifestations of periodontal disease and an abnormal healing response (Tatsumi et al., 2021). Smokers have a deeper probing depth and more gingival recession in their mouths than never-smokers, resulting in increased loss of bone, teeth, and dental implants, as observed in dental clinics (Albandar et al., 2000). Tobacco use is often preceded by negative health effects in the oral cavity, illustrating the importance of manag-

ing tobacco-related risk factors for dental practitioners. Thus, it is imperative to implement a safe, natural, and readily available smoking counter effect, such as vitamin C, to improve the cellular environment and dynamic behavior for more favorable clinical outcomes. Further investigation into the cellular level of tobacco smokers, the effect of topical vitamin C application, and its implementation in a clinical management protocol for these individuals is recommended.

6. Conclusion

Adverse harmful effects of tobacco smoking on human gingival fibroblasts was confirmed through results of Reverse Transcription Polymerase Chain Reaction on extracellular matrix construction markers, proliferation, and adhesion as integral components. In addition, this study emphasized the non-negligible effects of vitamin C on gingival fibroblast activity to reduce this negative effect of smoking. In the future, the duration and method of administering vitamin C should be determined to achieve maximum benefits. Furthermore, it would be beneficial to investigate the topical application of vitamin C for the clinical treatment of oral diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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