



ORIGINAL ARTICLE

Nicotine reduces cell viability and induces oxidative stress in human gingival fibroblasts

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ABSTRACT

BACKGROUND

Nicotine, as the main component of cigarettes, is known to interfere with the proliferation of human gingival fibroblasts (HGFs) and can trigger oxidative stress. This study aimed to analyze the impact of nicotine on viability, expression of the antioxidant Nrf2, levels of the product of oxidative stress malondialdehyde (MDA), and the migration capacity of HGFs.

METHODS

An experimental laboratory study used fibroblasts isolated from healthy human gingiva. The cells were grouped into the non-treatment control group (NTC), the solvent control (SC), and the treatment groups, exposed to nicotine at various concentrations for twenty-four hours. Cell viability was assessed using the cell counting kit-8 (CCK-8), Nrf2 expression was examined using ELISA, MDA level was measured using an MDA kit, and migration capacity was assessed using a scratch assay. Statistical analysis used one-way Anova or Kruskal-Wallis test. A p-value of <0.05 was expressed statistically significant.

RESULTS

The Cell viability was substantially reduced in the nicotine group compared to the untreated group, accompanied by changes in cell morphology. In contrast, Nrf2 expression increased significantly ($p=0.010$) in the 5 mM nicotine group compared with the control group. The MDA levels were not significantly distinct across groups ($p=0.056$). Cell migration was delayed significantly in the 5 mM nicotine group at 72 hours after scratching compared to the control group.

CONCLUSION

Nicotine decreased HGFs viability and increased Nrf2 expression significantly in a dose-dependent manner. Nicotine at 5 mM concentration did not alter MDA levels but delayed cell migration.

Keywords: Nicotine, gingival fibroblast, cell viability, MDA, Nrf2, cell migration

INTRODUCTION

Smoking increases the risk of specific illness.⁽¹⁾ Nicotine, aldehydes, hydrogen cyanide, carbon monoxide, and nitrosamines are just a few of the many compounds in cigarette smoke.⁽²⁾ According to estimates from the World Health Organisation, there are currently 1.3 billion smokers worldwide, and smoking-related deaths account for about 8 million deaths annually.⁽³⁾ In 2023, around 28.6 percent of the population aged 15 years and above in Indonesia were smokers.⁽⁴⁾

Most of the detrimental health effects of smoking, including addiction, are caused by nicotine.⁽⁵⁾ Oral health issues caused by nicotine addiction include candidiasis, leukoderma, leucoplakia, precancerous lesions, ulcers, halitosis, taste abnormalities, and cancer. Nicotine-related disorders of the periodontal tissue include deep gingival pockets, subgingival plaque, loss of attachment of connective tissue, loss of alveolar bone, periodontal disease, dental disorders characterized by foci of caries, and discoloration and loss of teeth.⁽⁶⁾ Nicotine and numerous anaerobic bacteria have been found on the surfaces of tooth roots in periodontal tissues, according to *in vitro* research.^(7,8) Furthermore, through reducing cell viability, nicotine has been demonstrated to modify several of the functions of cytotoxic human periodontal ligament fibroblasts.⁽⁹⁾ *In vitro* studies reported that high application of nicotine and cotinine have negative impact on human gingival fibroblasts (HGFs) cell adhesion and proliferation.⁽⁵⁾ The host immune response causes most periodontal tissue damage, involving leukocytes, complement, and reactive oxygen species (ROS).^(10,11) Once the level of damage surpasses the cell's ability to repair, it will induce permanent cell cycle arrest and changes in secretory phenotype reminiscent of the aging response.⁽¹²⁾

Free radicals can lead to oxidative stress by causing an imbalance between oxidants and antioxidants, possibly damaging cells.⁽¹³⁾ Free radicals boost lipid peroxidation, which then produces malondialdehyde (MDA).⁽¹⁴⁾ The serum MDA profile is an indicator of free radicals-induced cellular damage.⁽¹⁵⁾ One study revealed that patients with periodontitis had greater salivary MDA levels than healthy controls.⁽¹⁶⁾

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is part of a critical system to prevent ROS-related tissue

damage.⁽¹⁷⁾ This key transcription factor regulates antioxidant expression and is downregulated in neutrophils from patients with advanced periodontitis.^(17,18) According to reports, a deficit in Nrf2 can hasten osteoclastogenesis and heighten oxidative damage. In contrast, Nrf2 activation protects periodontal tissues from oxidative stress, periodontitis, and alveolar bone destruction.⁽¹⁹⁾

Fibroblasts, the primary mesenchymal cells of adult tissue, generate the intercellular substance's glycosaminoglycans and glycoproteins in addition to collagen, reticular, and elastic fibers.^(20,21) Fibroblasts are the leading players in wound healing because they are more active in synthesizing of matrix components in response to wounds by multiplying and enhancing fibrinogenesis.⁽²²⁾ Human gingival fibroblasts, as the most prevalent cell group in the oral mucosa, are more capable than skin fibroblasts of repairing wounds without leaving scars through adhesion, proliferation, and migration.⁽²¹⁾

Based on previously described research, nicotine can disrupt gingival fibroblasts and cause periodontitis. Several investigations have been conducted to evaluate the impact of nicotine on various cell groups; nevertheless, the impact of nicotine dosage on cell function is debatable. For this reason, it is necessary to elaborate on the effects of nicotine exposure in gingival fibroblasts by analyzing the cell viability, cell migration activity, and level of oxidative stress, including MDA level and Nrf2 expression.

METHODS

Research design

This investigation was an *in vitro* laboratory experiment with post-test design and was conducted at the Laboratory of Stem Cell Research Centre Universitas YARSI between August 2021 and April 2022.

Cell culture

Fibroblasts were taken from gingival tissue donors, particularly the third molars that matched the inclusion criteria. After extraction, to assure preservation and prevent cell damage, the extracted gingival tissue was put in a 50 mL tube filled with Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco). Gingival tissue samples were isolated using enzymatic disaggregation techniques and primary explants.

HGFs growth was observed from digested explant tissue by cold incubation overnight using dispase II. Cells were cultivated in Eagle's modified Dulbecco medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin B from Gibco in an incubator at a temperature of 37°C and 5% CO₂. Cells were cultured until in sufficient quantity for the following experiments.

HGFs were grouped into the non-treatment control group (NTC), consisting of cells receiving only complete medium (DMEM + 10% FBS + 1% antibiotics- antimycotic); the solvent control group (SC), consisting of cells receiving complete medium and 1.2% ethanol; and the nicotine treatment groups receiving complete medium, 1.2% ethanol, and exposed to nicotine at various concentrations. Each experiment was conducted in triplicate with two repetitions at different times.

Examination of cell viability

Cells were cultured into 96-well plates at a quantity of 5×10^3 cells per well in complete medium with five repetitions at each nicotine concentration. To evaluate cell viability, the cells were exposed to varying doses of nicotine (2.5 mM, 5 mM, 10 mM, and 20 mM) for 24 hours once a significant number of cells had been obtained. Three time periods (0, 24, and 48 hours) were used to assess the viability of HGFs using the CCK-8® (cell counting kit). The compound 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; (SIGMA) was analysed at 450 nm using a spectrophotometer.

Examination of Nrf2 Expression

HGFs were cultured in a 6-well plate at 105 cells/well. After reaching confluence, the cells were exposed to nicotine at 2.5 and 5 mM and incubated for 24 hours at 5% CO₂ and 37°C. The examination procedure was carried out according to the protocol contained in the ELISA kit (My BioSource; MBS1602823).

Examination of MDA

HGFs were grown in 12-well plates at 5×10^4 cells/well. After the number of cells was sufficient, they were exposed to nicotine at 2.5 and 5 mM and incubated for 24 hours at 5% CO₂ and 37°C. After 24 hours, HGF were harvested and examined for MDA levels based on the MDA kit protocol (SIGMA ALDRICH; MAK085).

Examination of cell migration capacity

HGFs were cultured at density of 2×10^4 cells/well in 24-well plates. Using a 1 mL pipette tip, a scratch was produced on the cell layer once the cells had reached 95% confluence, severing the connection between the cells. Then the HGFs were stimulated with nicotine at three different concentration levels for 24 hours. HGF migration capacity was evaluated by measuring the open area due to scratching using micrographs and an Evos XL Core microscope. Open area measurements by micrograph were performed serially for 24, 48, and 72 hours. Images of the test samples were taken in the same area. The open area due to the scratch assay was measured using T-Scratch software.

Data analysis

The data analysis was done with SPSS 26.0. First, the acquired data were examined for homogeneity and normalcy. The one-way ANOVA test was used to assess the normality and homogeneity of the data, and the Least Significant Difference (LSD) test was then used to ascertain the difference between the treatment groups. The Kruskal-Wallis test was used to assess non-normality and non-homogeneous data, followed by the Mann-Whitney test to examine variations in group averages. $P < 0.05$ was designated as the statistical significance threshold.

Ethical clearance

Patients' informed permission was obtained, and the study was approved by the local ethical commission (KEPK Universitas YARSI) under No. 247/KEP-UY/BIA/VII/2021.

RESULTS

Cell viability

Following a 24-hour exposure to nicotine, the number of viable HGFs decreased in a manner that was proportionate to the rise in nicotine concentration (Figure 1).

Human gingival fibroblast viability in the cell groups that received 2.5, 5, and 10 mM nicotine decreased significantly compared to the NTC group based on the ANOVA test ($p=0.000$). Nicotine at a concentration of 20 mM showed toxicity for HGFs, as seen from the death of nearly all HGFs after exposure to 20 mM nicotine.

Microscopic examination of cells in the NTC group and the 2.5 mM nicotine group showed fusiform cell morphology, fine cytoplasmic granules, and a large nucleus at the cell center. Meanwhile, HGFs at nicotine concentrations of 5

and 10 mM showed morphological changes to round, irregular cytoplasm without visible nucleus (Figure 2). In addition to the morphological differences, the NTC and nicotine groups differed in cell density.

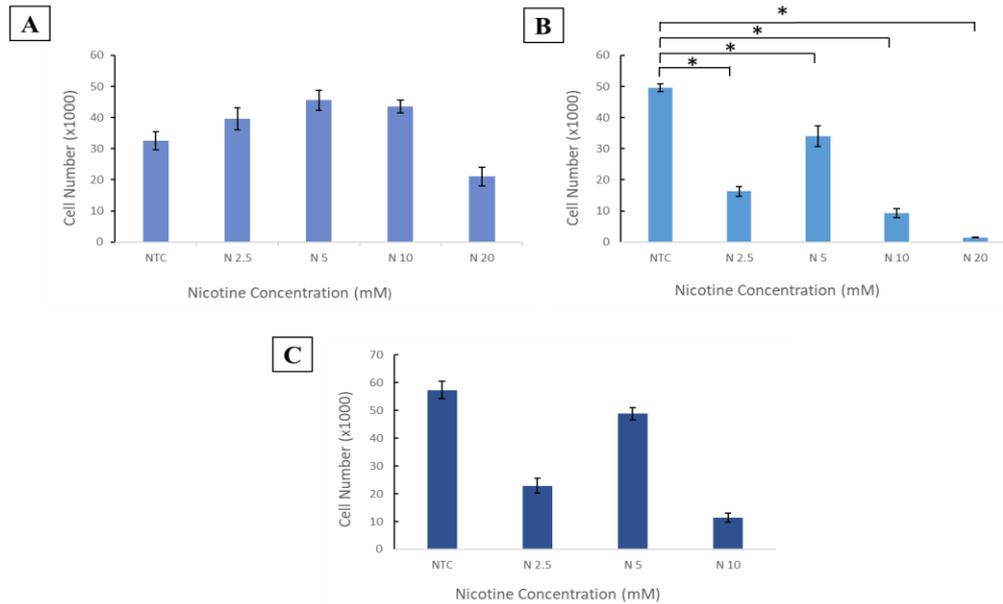


Figure 1. Effect of varying nicotine concentrations on HGFs cell viability (seeded on 96-well culture plates at a density of 5×10^3 cells/well).

(A) 0 hours of nicotine exposure; (B) 24 hours of nicotine exposure; (C) 48 hours of nicotine exposure. Data presented as mean \pm SD and analyzed by One-way ANOVA with LSD post-analysis; * indicates $p < 0.05$; NTC: non-treatment control; N 2.5: 2.5 mM nicotine; N 5: 5 mM; N 10: 10 mM nicotine; N 20: 20 mM nicotine.

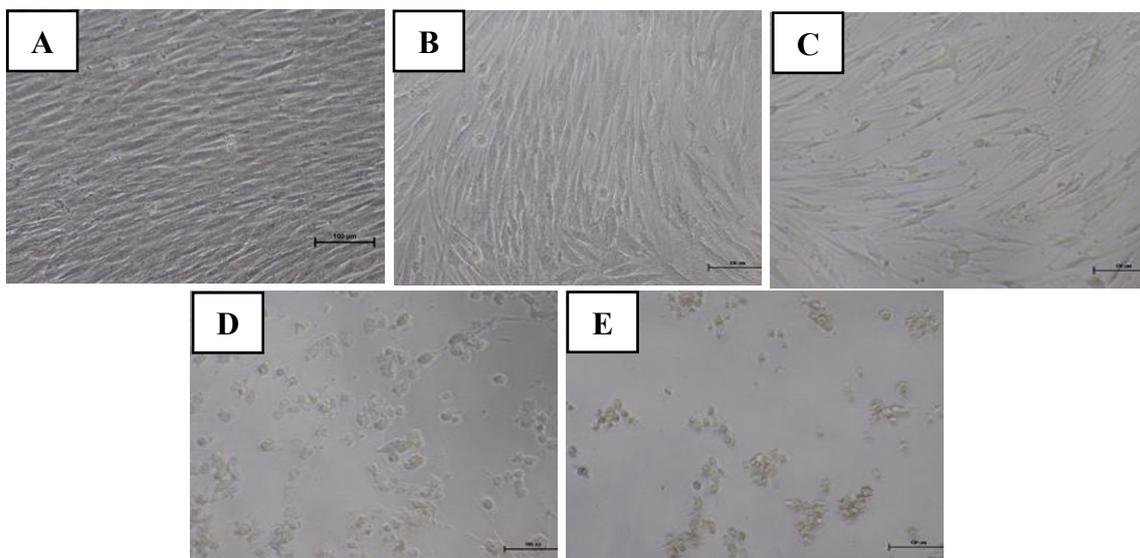


Figure 2. Appearance of human dermal fibroblasts (HGFs) after 24 hours of exposure to nicotine in various concentrations at a magnification of 100 x. (A) Non-treatment control HGFs; (B) HGFs exposure to 2.5 mM nicotine; (C) HGFs exposure to 5 mM; (D) HGFs exposure to 10 mM, (E) HGFs exposure to 20 mM.

Nrf2 expression

Our study demonstrated a significant discrepancy in Nrf2 expression between treatment groups (ANOVA; $p=0.010$). Post hoc tests using LSD showed that Nrf2 expression increased significantly in the 2.5 and 5 mM nicotine groups compared to the NTC and SC groups (Figure 3).

MDA levels

Compared to the NTC and SC groups, there was a tendency of increased MDA levels in the HGFs group receiving nicotine (Figure 4). The highest MDA levels were seen in the 5 mM nicotine group compared to the other groups, although the Kruskal-Wallis test did not show a significant distinction ($p=0.056$).

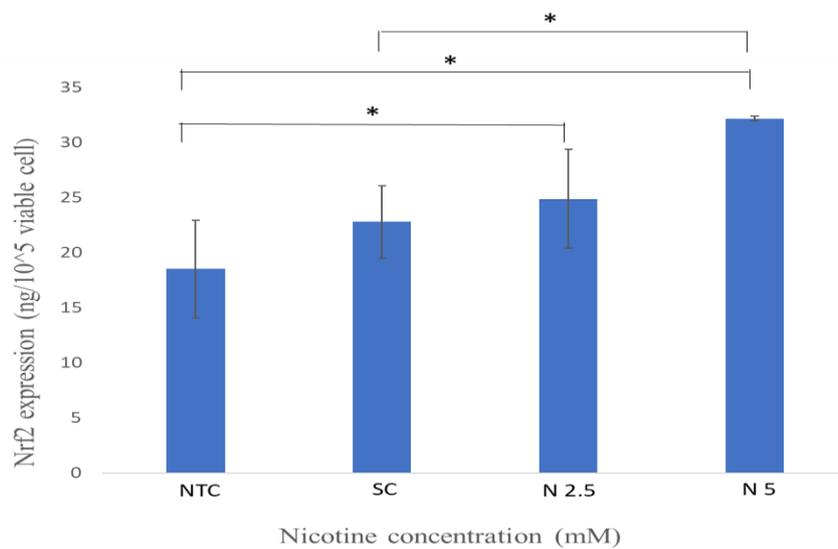


Figure 3. Expression of Nrf2 in HGFs after 24 hours of nicotine exposure. Cells seeded in 6-well culture plates at a density of 10^5 cells/well.

Data presented as mean \pm SD and analyzed by One-way ANOVA with LSD post-analysis; * indicates $p < 0.05$; NTC: non-treatment control; SC: solvent control; N 2.5: 2.5 mM nicotine; and N 5: 5 mM.

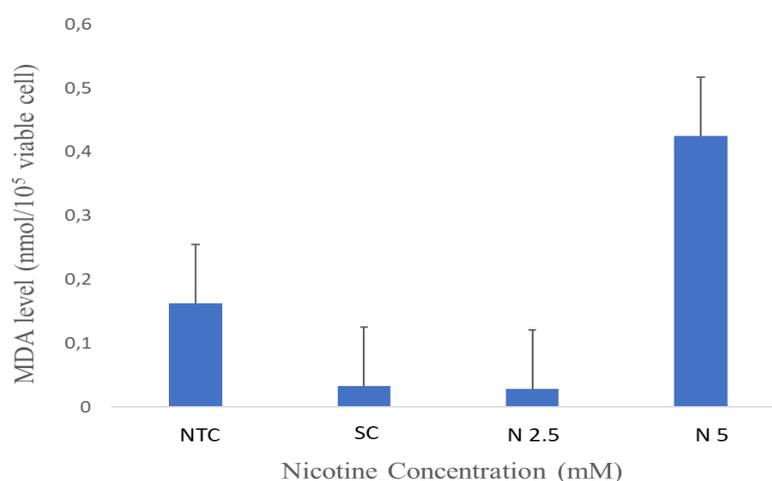


Figure 4. Levels of MDA in HGFs after 24 hours of nicotine exposure. Cells seeded in 12-well culture plates at a density of 5×10^4 cells/well.

Data presented as mean \pm SD and analyzed by the Kruskal-Wallis test ($p=0.056$); NTC: non-treatment control; CS: solvent control; N 2.5: 2.5 mM nicotine; and N 5: 5 mM.

Cells migration capacity

There was no visible difference in the size of the open areas between the HGF groups at 0 and 24 hours after etching. At 48 hours of observation, the open area in the HGF group receiving nicotine was relatively wider than in the control group (Figure 5). However, quantifying the area by T-Scratch software did not produce a significant difference in area. At 72 hours of observation, the open areas in all HGF groups had closed, except in the HGFs group exposed to 5 mM nicotine. The Kruskal-Wallis statistical test supports these findings; a significant difference in open area was seen at 72 hours of observation ($p=0.00$) between the 5 mM HGF nicotine group and the control group (Figure 6), indicating a decrease in HGF migration capacity in this group.

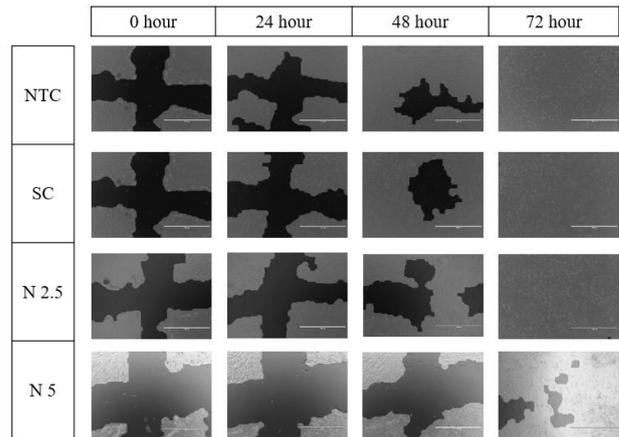


Figure 5. Visualization of HGFs migration after scratch assay using T-Scratch software. Observations were made at 0, 24, 48, and 72 hours after scratching. Pictures are taken at the same observation area each time. NTC: non-treatment control; CS: solvent control; N 2.5: 2.5 mM nicotine; and N 5: 5 mM.

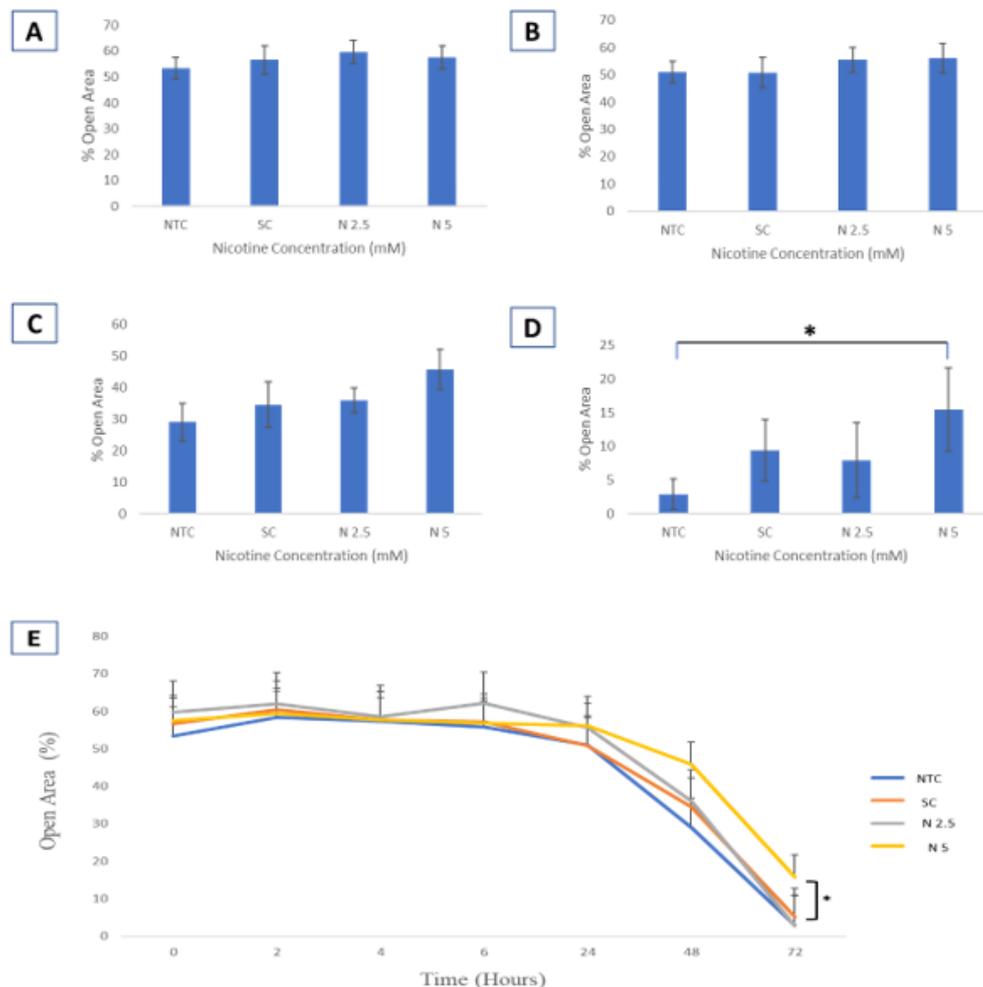


Figure 6. The migration capacity of HGFs after 24 hours of nicotine exposure. Cells seeded in 24-well culture plates at a density of 2×10^4 cells/well. (A) 0 hours of observations; (B) 24 hours of observation; (C) 48 hours of observation; (D) 72 hours of observation; (E) 0-72 hours of observation.

Data presented as mean \pm SD and analyzed by the Kruskal-Wallis test with Mann Whiney post-analysis, * indicates $p < 0.05$. NTC: non-treatment control; CS: solvent control; N 2.5: 2.5 mM nicotine; and N 5: 5 mM.

DISCUSSION

Smoking is a significant risk factor for periodontal disease of external origin. Smokers are at high risk for the onset and progression of periodontal disease. The classification and extent of periodontitis correlated with smoking status and the number of cigarettes smoked. Wound healing after periodontal treatment is influenced by smoking, because smokers tend to respond less well to non-surgical and surgical periodontal treatment than non-smokers. Nicotine is one of the main detrimental components of tobacco and is considered the main addictive chemical of tobacco products. In vitro studies have reported the injurious effects of nicotine on various cells.⁽²³⁾

In the present in vitro study, we examined the effects of nicotine at the cellular level on HGFs, a group of cells that plays an essential role in the healing of damaged tissue in periodontal disease. The parameters of this study were viability, MDA level, Nrf2 expression, and migration capacity of HGFs after nicotine exposure. We applied two nicotine concentration levels, 2.5 mM and 5 mM, to assess Nrf2 and MDA levels, and HGFs migration capacity, considering that nicotine doses greater than 5 mM are already toxic and interfere with the validity of the assessment of these three biomarkers.

We observed that cell viability was inhibited by nicotine exposure in dose- and exposure time-dependent ways. At the nicotine concentration of 10 mM, the fibroblasts showed some toxic effects, while nicotine concentrations of 5 mM and 2.5 mM did not show a toxic effect on the fibroblasts. The research that we have done demonstrates the irreversible cytotoxic effect of 10 mM of nicotine on human gingival fibroblasts. In comparison, nicotine concentrations of 2.5 mM and 5 mM showed a cytotoxic effect that may interfere with HGFs mitotic activity, indicating a cell aging process. In their research, Hardin et al.⁽²⁴⁾ reported that high nicotine concentrations can interfere with cell proliferation and cause exaggerated senescence, a biological process related to cell longevity and inability to replicate.

Many factors influenced this in vitro research, such as nicotine concentration and length of exposure. Interestingly, the in vitro study conducted by Dinos et al.⁽²⁵⁾ reported a significant reduction of 49% in wound repopulation after four days of exposure to 4 mM nicotine and of 67% after six days with as little as 1 mM nicotine. In

contrast, Lallier et al.⁽²⁶⁾ reported no effect of up to 1 mM for 5 days. Furthermore, higher concentrations showed cell morphology associated with either cell death or aging, whereas our study showed cell death at 10 mM nicotine dose on day 2. Another in vitro study found that nicotine had a significant effect on cell viability and migration.⁽²⁷⁾ Meanwhile, Esfahrood et al.⁽⁵⁾ reported a decrease in gingival fibroblast cell viability and toxicity at concentrations of 10^{-3} to 2×10^{-2} M after 24 and 48 hours of nicotine exposure.

Our study showed that Nrf2 expression significantly increased in the HGFs group receiving nicotine at all concentrations compared with the NTC group. Nrf2 expression was not significantly different in the 2.5 mM nicotine concentration group compared to the SC group but was significantly different in the 5 mM nicotine concentration group. This shows that nicotine increases Nrf2 expression in HGFs and that the ethanol solvent does not affect HGFs.

The increase in Nrf2 expression occurs because Nrf2 is a transcription factor that is an essential regulator for the induction of antioxidant enzymes and fighting oxidative stress. Oxidant substances such as nicotine, to which periodontal tissues are exposed, will increase the formation of ROS in the cytoplasm, thereby activating transcription factors that are linked to inflammation through the Kelch-like ECH-associated protein-1 (KEAP1) pathway, which ultimately activates the Nrf2 transcription factor in the cytoplasm, after which Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) of several antioxidant genes, thereby activating their transcription.⁽¹⁷⁾ The results of this study are in accordance with research conducted by Park and Yoon,⁽²⁸⁾ which stated that the higher the level of oxidative stress generated, the higher the Nrf2 expression produced.

Research by Xue et al.⁽²⁹⁾ shows that exposure of human umbilical vein endothelial cells (HUVECs) to cigarette extract activates Nrf2 expression. In periodontal disease, increased expression of Nrf2 can prevent periodontal tissue from developing periodontitis and alveolar bone damage,⁽¹⁹⁾ and Li et al.⁽³⁰⁾ showed a decrease in Nrf2 expression in the nucleus and cytoplasm of the diabetic periodontitis group compared with the periodontal tissue control group.

Malondialdehyde (MDA) is one of the most widely used biomarkers of lipid peroxidation. The increased MDA concentration may result from reduced antioxidant production caused by nicotine. Lipid peroxidation of unsaturated fatty acids is an indicator of increased oxidative stress. Nicotine can induce the production of superoxide anions and hydroperoxides, which are the primary sources of free radicals and markers of oxidative stress. Compared to controls, MDA levels increased significantly in the saliva of tobacco-chewing subjects⁽³¹⁾ and active smokers.⁽³²⁾ MDA levels also increased in smokers with periodontitis compared to healthy control individuals.⁽³³⁾

Our study showed a tendency for MDA levels to increase in the HGFs group who received nicotine. However, the increase was not statistically significantly different from the NCT and SC groups. This is different from research by Kumar et al.,⁽³⁴⁾ which showed a significant increase in MDA levels in periodontitis due to nicotine. Khademi et al.⁽³⁵⁾ showed a non-significant increase in MDA levels in cells exposed to low nicotine concentrations. However, there was a significant increase in MDA levels at high nicotine concentrations compared to the controls. The non-significant results in our study may be in line with research by Nguyen et al.,⁽³⁶⁾ which showed that nicotine concentrations of 1 and 10 ng/mL did not significantly increase MDA levels in periodontal ligament fibroblasts (PDLFs) after 2 hours of exposure. Meanwhile, the opposite results were shown after 24 hours of exposure. This explains why the oxidative stress process occurs over time. The length of exposure to nicotine influences the detectability of the increase in MDA, therefore further tests need to be carried out with nicotine exposure for more than 24 hours. Nguyen et al.⁽³⁶⁾ also stated that the MDA levels were not significantly different in their study, indicating that nicotine may not affect lipid peroxidation in PDLFs.

In our study, increased Nrf2 expression activates endogenous antioxidants that are relatively sufficient to reduce ROS, such that ultimately MDA, a product of lipid peroxidation, does not increase significantly. Of course, this requires proof with further research to assess the expression of endogenous antioxidant biomarkers such as glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), or catalase.

Cell migration plays an essential role in physiology, development, and disease-related

processes. Cells must be able to respond to external signals to migrate according to needs such as growth, immune response, or repair of tissue damage. Failure to migrate properly can cause various disorders, such as defects in the formation of nervous tissue, immunodeficiency, or wounds that do not heal. In the initial healing phase of an open wound, resident fibroblasts proliferate and migrate from the wound edges to form granulation tissue that will close the wound. Research shows that smoking and nicotine itself can interfere with the migratory ability of fibroblasts.⁽³⁷⁾

The migration activity test showed no significant difference in the open area between treatment groups at 24 hours and 48 hours. However, the open area in the cell group that received 5 mM nicotine appeared to be wider than in the other groups. A significant slowdown in migration was visible after 72 hours of observation in HGFs exposed to nicotine at a concentration of 5 mM.

Silva et al.⁽³⁸⁾ showed that nicotine in small doses, equal to or less than 9.6 µg/mL, stimulates the proliferation and migration of gingival fibroblasts. Fibroblast migration was inhibited when administering nicotine at concentrations starting from 16 µg/mL. Chen et al.⁽⁹⁾ stated that nicotine is a cytotoxic agent for cementoblasts, inhibiting cell growth and migration. This inhibition was influenced by the nicotine concentration and the length of exposure to nicotine in the study of Esfahrood et al.,⁽⁵⁾ in that low concentrations of nicotine and cotinine caused a reduction in cell adhesion. High nicotine and cotinine concentrations affected cell adhesion and HGF proliferation. The cell migration process previously influenced the inhibition of adhesion and proliferation, so that it would have an impact on inhibition of the wound healing process.

For cells to migrate, they must modify the actin cytoskeleton and extend long lamellipodia at their migration front. During migration, cells must also recognize, via integrin receptors, specific molecules in the extracellular environment to allow attachment to extracellular matrix molecules. An *in vitro* study on endothelial progenitor cells showed that smoking-related ROS suppressed the Akt/eNOS/NO pathway and decreased expression of integrins and VEGF. This, in turn, contributes to a decrease in the migration ability of endothelial cells and tube formation, which are important steps in angiogenesis.⁽³⁹⁾

Another important mechanism involved in cell migration is the proteolytic degradation of extracellular matrix components required to allow cells to pass through this environment. Fibronectin is an essential extracellular matrix (ECM) glycoprotein critical in development, wound healing, and angiogenesis. Plasma fibronectin is secreted by hepatocytes and circulates in the blood, whereas cellular fibronectin is secreted and stored in the ECM by various cell types, including fibroblasts. Fibronectin is secreted as a soluble dimer and has been shown to form insoluble fibers upon integrin-mediated cell surface binding and force application. In addition to providing adhesive structural support, which many cell surface receptors recognize, fibronectin also forms a template to which other ECM proteins and soluble growth factors can bind and assemble.⁽⁴⁰⁾

Xue et al.⁽²⁹⁾ stated that fibronectin promotes endothelial cell activation, survival, migration, and elongation. Fibronectin interacts with integrins via the RGD (Arg–Gly–Asp) motif to form fibrils, and the complex then binds to focal adhesion kinase (FAK) or actin to carry out various cellular processes. Xue's research shows that cigarette extract reduces the formation of fibronectin fibrils and disrupts actin filaments in HUVECs.

Our study has limitations in exposure time and range of nicotine concentration. In this study, nicotine disrupted cell viability and induced oxidative stress in HGFs. This study provides additional information about molecular and cellular behavior in smokers, which may be helpful as a basis for future studies of new treatment strategies for smokers.

CONCLUSIONS

Nicotine immensely decreased the viability of HGFs and escalated the expression of Nrf2 in a dose-dependent manner. Nicotine did not increase MDA levels but restrained the migration of HGFs. Further research that can be recommended is to extend the duration of nicotine exposure to more than 24 hours to evaluate its effect on MDA and ROS formation and its impact on endogenous antioxidant enzymes.

Conflict of interest

The Authors declare no conflict of interest.

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Author Contributions

SA, RSH and IK conducted the experiment, WS, RSH, YS designed the experiment and did data analysis. All authors contributed equally to the writing of the manuscript. All authors have read and approved the final manuscript.

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Data Availability Statement

The data used to support the findings of this study is available from the corresponding author upon request.

Declaration of Use of AI in Scientific Writing

Nothing to declare.

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