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Effect of Nicotine on Attachment and Proliferation of Human Gingival Fibroblasts on Zirconia and Titanium Surfaces

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Abstract

Background and objectives: This study aimed to evaluate the effect of nicotine on the biological behavior of human gingival fibroblasts on titanium and zirconia surfaces.

Materials and methods: A total of 124 Polished titanium and zirconia disc-like samples, each with a diameter of 9 mm and a thickness of 2 mm, were prepared. A contact surface profilometer was used to measure surface roughness with Ra and Rz parameters. HGFs were treated with different concentrations of nicotine. Then, their adhesion, morphology, viability, and proliferation were measured using the methyl thiazolyl tetrazolium (MTT) assay and scanning electron microscopy (SEM) at 24 and 72 hours. A t-test analysis was used to compare the surface roughness of the specimens. Data were reported as mean ± SE. Graph-Pad Prism software (version 9, USA) was used for a one-way analysis of variance (ANOVA) followed by a Tukey post hoc analysis. A P-value of < 0.05 was considered significant.

Results: The proliferation and attachment of HGF cells to the titanium and zirconia surfaces were significantly reduced by increasing nicotine concentration up to the cytotoxic range (2.5 and 5 mM) or prolonging the amount of time cells are exposed to nicotine *($P < 0.05$)*. The difference between the zirconia and titanium groups was not statistically significant *($P > 0.05$)*.

Conclusion: Nicotine cytotoxicity was concentration and time-dependent. There was no significant difference between the zirconia and titanium groups. Considering the limitations of this study and the conflicting results reported by previous studies, additional research is required to determine the superiority of these two materials, particularly in patients who smoke.

Keywords: Cell adhesion, Fibroblasts, Gingiva, Nicotine, Titanium, Zirconium.

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1. Introduction

Smoking is extremely common in many populations around the world, and it is one of the most important risk factors for periodontal disease (1-3). Cigarette smoke contains more than 7,000 chemical substances (4). Nicotine is one of the toxic agents in cigarette smoke. It can increase the secretion of inflammatory cytokines (e.g., TNFα and IL6) in the gingival tissue (5). Many smokers experience tooth loss and need oral rehabilitation with dental implants to return to a functional masticatory system. However, various studies indicate that smoking is a potential risk factor for dental implant failure (5,6). A 10-year longitudinal study suggested that implant failures are significantly higher in smokers than non-smokers (7). With the negative biological effects of smoking in mind, special considerations for implant treatments in smokers are required (8-10).

Stable soft tissue attachment to the abutment (i.e., the efficient

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seal between the implant's osseointegrated part and the surrounding soft tissue notably affect the implant's long-term survival. Human gingival fibroblasts (HGFs) play a crucial role in the repair of epithelial and connective tissues following implant placement. They produce extracellular matrix components such as collagen (11). In addition, they act against bacterial penetration into peri-implant soft tissue, thereby preventing clinical complications caused by microbial inflammation (e.g., soft tissue recession and marginal bone loss) (12). It seems that the biological behavior of HGFs and epithelial cells is influenced by the surface features of abutments and the type of abutment material (13-15). Atsuta et al. compared the proliferation, apoptosis, and adhesion of oral epithelial cells on rough and machined titanium surfaces (16). The results showed higher proliferation and adhesion and less cell apoptosis on machined surfaces. Also, cells cultured on machined surfaces expressed higher amounts of cell adhesion proteins. Until now, titanium has been widely used as a material for implant and abutment production. In recent years, zirconia has been introduced as an alternative material to titanium, particularly for the abutments of esthetic zone implants (9-17). An *in vitro* study reported that HGFs that were cultured on Laser-Lok surfaces displayed more mature morphology and had higher proliferation and differentiation than those on zirconia and titanium surfaces. However, the differences between zirconia and titanium surfaces were insignificant (8).

Previous studies have shown the adverse effect of nicotine on HGF cell proliferation and adhesion to various surfaces. Esfahrood et al. indicated that nicotine at low concentrations reduced the 'HGFs' initial adhesion to dental root surfaces (18); however, the cells proliferated successfully after 48 hours. In contrast, highly concentrated nicotine had an adverse effect on both cell adhesion and proliferation. Nicotine's role in altered fibroblast cell adhesion to the root surface was also reported by Martinez et al. (19). Another *in vitro* investigation reported that rather than high concentrations, low concentrations of nicotine could stimulate the HGFs' adhesion to the plastic surface (20). Considering the importance of HGFs' function in peri-implant soft tissue healing and maintenance, we aimed to evaluate the adhesion and proliferation of HGFs on zirconia and titanium surfaces treated with nicotine at various concentrations and durations.

2. Materials and Methods

2.1. Titanium and zirconia specimen preparation

In this *in vitro* experimental study, a total of 124 titanium (TiAl6V4) and zirconia (Zr) discs with 9 mm diameter and 2 mm thickness were prepared. (Figure 1)

2.2. Surface roughness evaluation

The surfaces of these samples were similar in having equal surface roughness. The surface roughness was evaluated (n = 10 in each group) using a surface roughness tester (TR200, SaluTron GmbH). Each disc was triple-measured in different areas without overlaps. Ra (average surface roughness) and Rz (average depth measures of the surface roughness) indicators were noted.

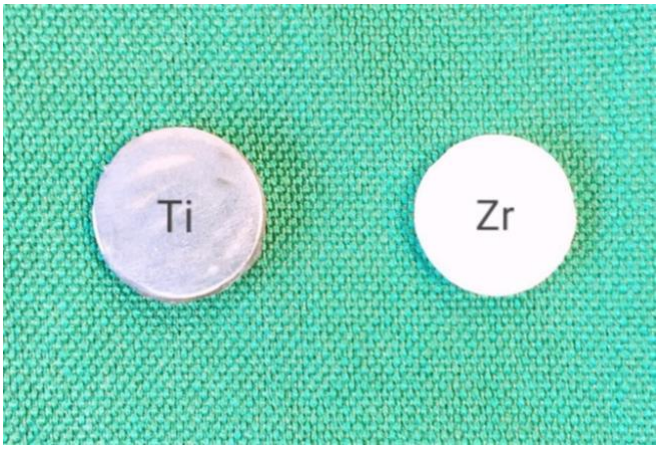

Figure 1. Titanium and zirconia discs.

2.3. Cell culture

Specimens with similar and uniform surface roughness were sterilised with 70% ethanol, triple-rinsed with distilled water, and then autoclaved separately. HGF1 PI 1 (NCBI: C165) cells were collected from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin-streptomycin (GIBCO) and incubated with 5% CO2 at 37 °C in a 95% humidified atmosphere. Titanium and Zirconia discs (n = 6 in each group) were placed in sterile, non-treated polystyrene 48-well culture plates for 24 and 72 hours of evaluation, respectively. A total of 104 cells (passage number = 5) were seeded on each disc. Culture mediums (500 µL) containing different concentrations of nicotine (0 (control group), 1 µM, and 1, 2.5, and 5 mM) were added to the wells. The concentrations were chosen after reviewing the articles from Holiday et al.'s systematic review and also based on our previous studies (18, 21, 22, 23). Similar samples were also prepared in two 48-well culture plates without discs. All samples were transferred to the incubator and kept until the evaluation times.

2.4. Cell vitality and proliferation

After 24 and 72 hours, the 48-well culture plates were removed from the incubators, and the medium was aspirated entirely. After washing the cells with phosphate-buffered solution (PBS) twice, a 500 µL fresh culture medium containing 10% MTT dye solution (5 mg/mL) (without FBS and antibiotics) was added to each well. The culture plates were then incubated at 37 °C for three hours until purple formazan crystals formed. Following incubation, the medium was removed, and 200 µL of DMSO (dimethyl sulfoxide) solvent was added to dissolve the formazan crystals. 100 µL

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of the purple solution from each well was transferred to a 96-well plate to measure the spectrophotometric absorbance (optical density; OD) at 570 nm using an ELISA microplate reader (Anthos 2020). A wavelength of 620 nm was used as a reference. Primary cell attachments were evaluated and compared based on 24-hour tests of ODs, while cell proliferation rates were measured by comparing 72-hour test results to 24-hour ones.

2.5. Cell morphology

After 24 and 72 hours of incubation, the medium was aspirated, and the cells were washed with PBS. Cells' fixation was done in 2.5% glutaraldehyde (Sigma-Aldrich) for 24 hours. Then, the specimens (n = 3 in each group) were washed several times with deionised water (10 minutes) and a graded series of ethanol solutions (30%, 50%, 70%, 80%, 90%, and 100%) (10 minutes for each solution) for dehydration. Finally, the ethanol was removed, and the specimens were air-dried. An inverted microscope (Nikon) with 10× magnification was used to observe cells attached to polystyrene cultures. Cells attached to zirconia and titanium disks were assessed by SEM electron microscopy (Arya Electron Optics Co., Tehran, Iran). Before capturing the images with the SEM device, the specimens were coated with sputtered gold. The specimens were analysed at ×20, ×50, and ×100 magnifications.

2.6. Statistical analysis

All tests were repeated at least twice with similar results. Data were reported as mean ± SEM. A t-test analysis was used to compare the surface roughness of the specimens. Graph-Pad Prism software (version 9) was used for a two-way analysis of variance (ANOVA) followed by a Tukey post hoc analysis. A P-value < *0.05* was considered significant.

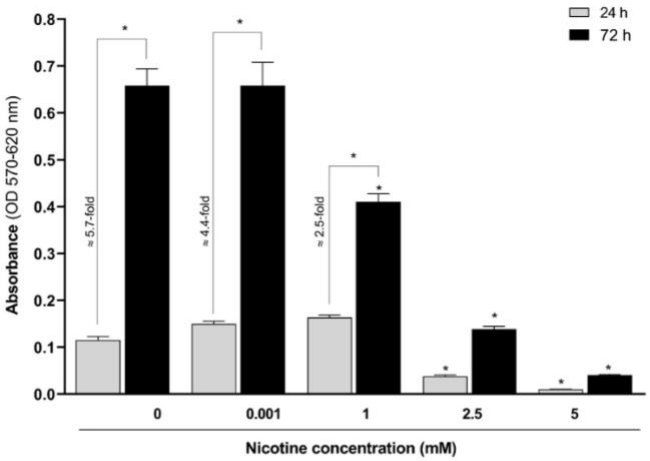
3. Results

3.1. Surface roughness

The Ra and Rz (µm) indices were 0.27 ± 0.06 and 1.54 ± 0.27 in titanium and 0.43 ± 0.07 and 2.21 ± 0.31 in zirconia discs. Although the surface roughness parameters (Ra and Rz) were both higher in zirconia than in titanium, the T-test analysis did not show a statistically significant difference between the groups ($P > 0.05$; 0.0973 for Ra and 0.1283 for Rz; t = 1.645, df = 11).

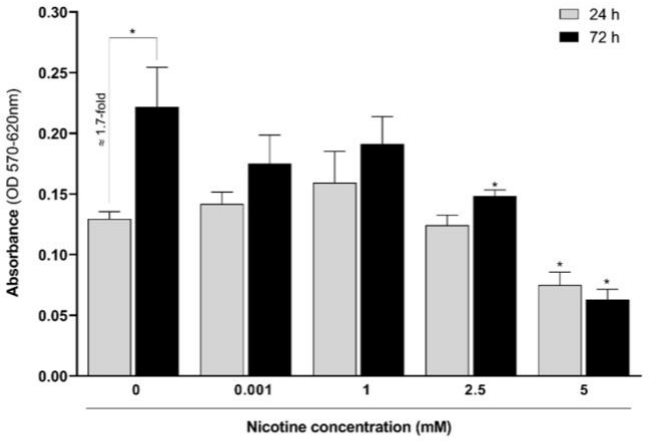
3.2. Cell adhesion and viability

Cell adhesion and viability were evaluated in zirconia, titanium, and polystyrene (culture plates without discs) at different nicotine concentrations (0.1 µM, 1 mM, 2.5 mM, and 5 mM) after 24- and 72-hour incubation periods.
Cell adhesion and vitality were significantly lower in 2.5 (0.04 ± 0.00) and 5 (0.01 ± 0.0) mM nicotine concentrations than in other concentrations in 24 h evaluations of polystyrene specimens ($P < 0.0001$, F = 130.9). The difference between 2.5 mM and 5 mM concentrations was not statistically significant (P = 09988, F = 130.9) (Figure 2). Only at the 5 mM nicotine concentration did the titanium (0.08 ± 0.01, P = 0.3520, F = 10.12) (Figure 3) and zirconia ([0.07 ± 0.01, P = 0.0951, F = 10.64) (Figure 4) groups' cell adhesion and vitality differ significantly from those of the control group.



	HGF Cell Attachment/Proliferation *(Mean±SEM)* on **Polystyrene surface** *in* presence *of* **Nicotine**				
	0	**1 µM**	**1 mM**	**2.5 mM**	**5 mM**
24 h	0.12±0.01	0.15±0.01	0.16±0.01	0.04±0.00	0.01±0.00
72 h	0.66±0.04	0.66±0.05	0.41±0.02	0.14±0.01	0.04±0.00

Figure 2. The effect of nicotine at different concentrations on cell adhesion and vitality percent in the polystyrene group is calculated and compared to the control group (*$P < 0.05$). The rate of proliferation is calculated and compared at two time points.



	HGF Cell Attachment/Proliferation *(Mean±SEM)* on **Titanium disks** *in the presence of* **Nicotine**				
	0	**1 µM**	**1 mM**	**2.5 mM**	**5 mM**
24 h	0.13±0.02	0.14±0.01	0.16±0.03	0.12±0.01	0.08±0.01
72 h	0.22±0.03	0.18±0.02	0.19±0.02	0.15±0.01	0.06±0.01

Figure 3. The effect of nicotine at different concentrations on cell adhesion and vitality percent in the titanium group is calculated and compared to the control group (*$P < 0.05$). The rate of proliferation is calculated and compared at two time

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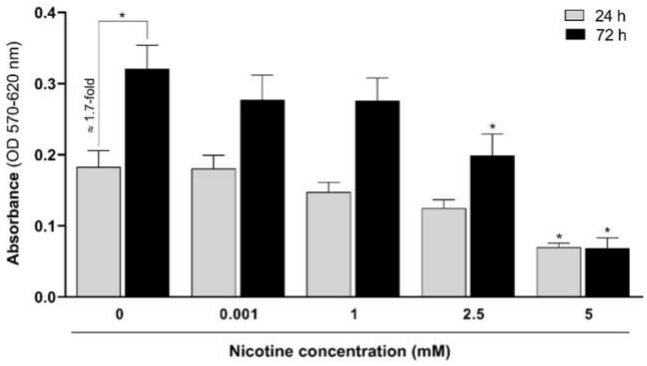
points.



	HGF Cell Attachment/Proliferation *(Mean±SEN)* *on* **Zirconia disks** *in the presence of* **Nicotine**				
	0	**1 µM**	**1 mM**	**2.5 mM**	**5 mM**
24 h	0.18±0.02	0.18±0.02	0.15±0.01	0.13±0.01	0.07±0.01
72 h	0.32±0.03	0.28±0.03	0.28±0.03	0.20±0.03	0.07±0.01

Figure 4. The effect of nicotine at different concentrations on cell adhesion and vitality percent in the zirconia group is calculated and compared to the control group (*$P < 0.05$). The rate of proliferation is calculated and compared at two time points.

Comparing polystyrene, titanium, and zirconia groups at 0.1 µM and 1 mM nicotine concentrations, cell adhesion and vitality differences were not statistically significant. However, at 2.5 [(0.15 ± 0.06 for titanium; $P = 0.0004$, F = 12.79) and (0.13 ± 0.03 for zirconia; $P = 0.0043$, F = 12.79)] and 5 [(0.08 ± 0.02 for titanium; $P = 0.007$, F = 15.98) and (0.07 ± 0.01 for zirconia; $P = 0.0011$, F = 15.98)] mM concentrations, the pairwise comparison showed significantly higher cell adhesion in the zirconia and titanium groups than in the polystyrene group. The difference between the zirconia and titanium groups was not significant (Figure 5).

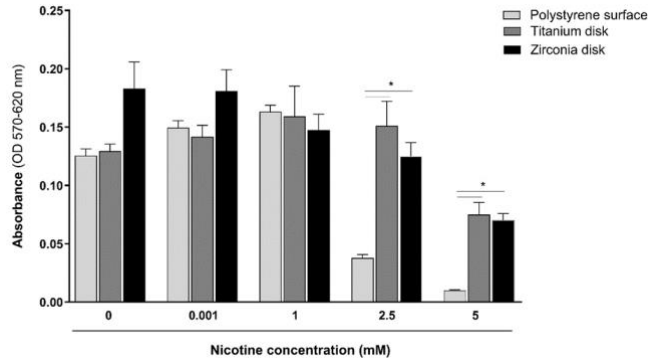


Figure 5. Cell adhesion and vitality in polystyrene, zirconia, and titanium groups after 24 hours of evaluation in various nicotine concentrations (*$P < 0.05$)

After 72 h of incubation, rather than in cells treated with a 1 µM nicotine concentration, the cells' vitality and adhesion to polystyrene were significantly lower in other concentrations than in the control group. The cells cultured on titanium (Figure 3) and zirconia (Figure 4) discs had significantly lower adhesion and were less vital in 2.5 [(0.15 ± 0.01for titanium; $P = 0.0287$, F = 10.12) and (0.20 ± 0.03 for zirconia; $P < 0.0001$, F = 10.64)] and 5 mM [(0.06 ± 0.01 for titanium; $P < 0.0001$, F = 10.12) and (0.07 ± 0.01for zirconia; $P < 0.0001$, F = 10.64)] nicotine concentrations. Among the polystyrene, titanium, and zirconia groups, the difference between the three groups was statistically significant at nicotine concentrations of 0.1 µM and 1 mM, in contrast to the 2.5 and 5mM concentrations. Pairwise comparisons of the groups revealed significantly higher cell adhesion in the polystyrene group than in the zirconia and titanium groups. In contrast, the difference between the zirconia and titanium groups was not significant (Figure 6).

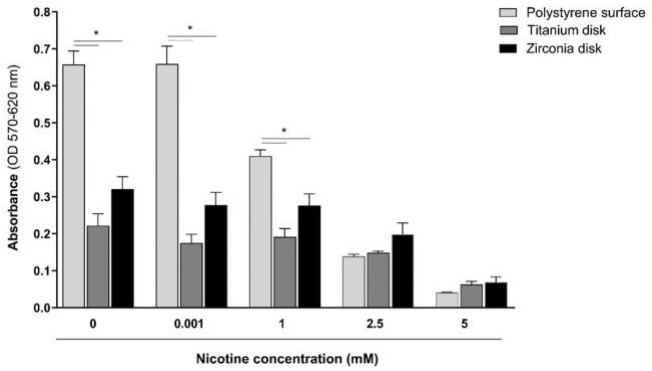


Figure 6. Cell adhesion and vitality in polystyrene, zirconia, and titanium groups after 72 hours of evaluation in various nicotine concentrations (*$P < 0.05$)

3.3. Cell proliferation

To evaluate the proliferation and the nicotine cytotoxicity, cell numbers at 24 and 72-hour time points were compared in each group. In polystyrene surface cultures, cell numbers were increased up to 5.7 ($P < 0.001$, F = 130.9), 4.4 ($P < 0.001$, F = 130.9), and 2.5 ($P < 0.001$, F = 130.9) fold at 0 (the control group), 1µM, and 1 mM nicotine concentrations, respectively. In comparison, there was no significant difference in terms of proliferation at 2.5 and 5 mM concentrations. (Figure 6) In the titanium and zirconia groups, cell numbers increased only in the control group [1.7-fold ($P = 0.0023$, F = 10.12 for titanium) ($P = 0.0021$, F = 10.64 for zirconia)], indicating a significant cytotoxic effect of nicotine at all concentrations. (Figure 3,4)

3.4. Cell morphology

Electron and light microscopy showed cell adhesion to all surfaces. In comparison to zirconia, cell adhesion was higher in titanium due to less surface roughness. In the polystyrene group, cells exhibited vacuolated cytoplasm and significantly

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lower mitotic activity when the nicotine concentration exceeded 1 mM. At concentrations of 2.5 and 5 mM, cellular apoptosis was evident (Figure 7). In the titanium and zirconia groups, along with the increase in nicotine concentration, cell numbers decreased, and more apoptotic cells were observed in both the 24- and 72-hour observations (Figure 8,9).

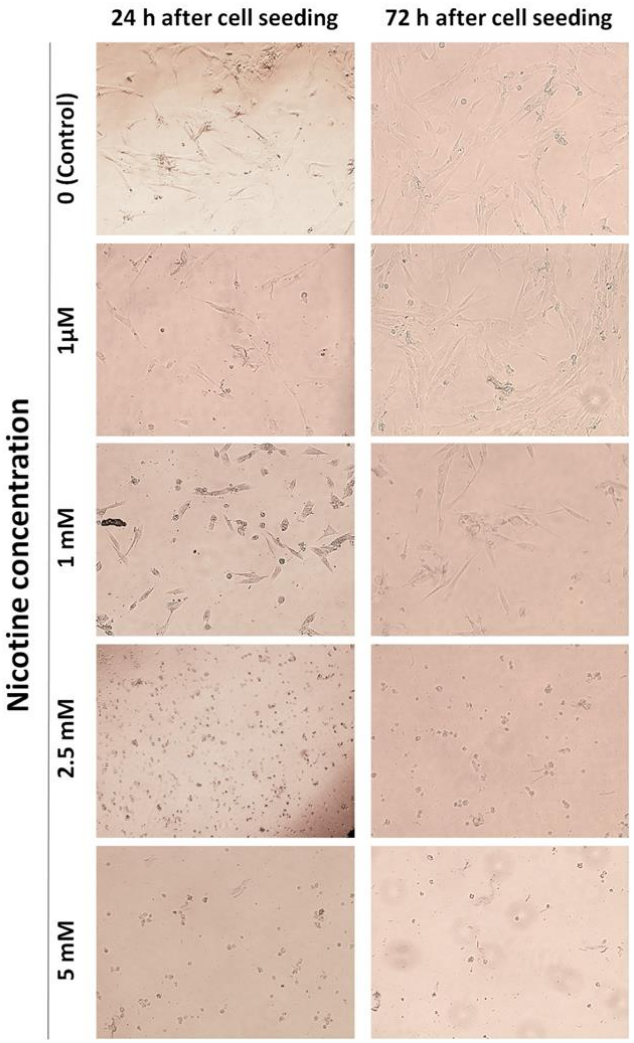

Figure. 7. Inverted microscope images (10x magnification) of attached HGF cells on polystyrene surfaces 24 and 72 hours after nicotine exposure at various concentrations.

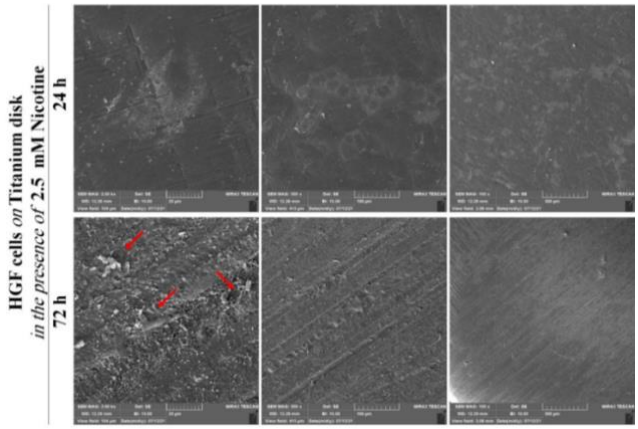

Figure 8. SEM images (20, 100, and 500 µm) of attached HGF cells on titanium surface, 24 and 72 h after exposure to a 2.5 mM concentration of nicotine; red arrows point at the apoptotic cells.

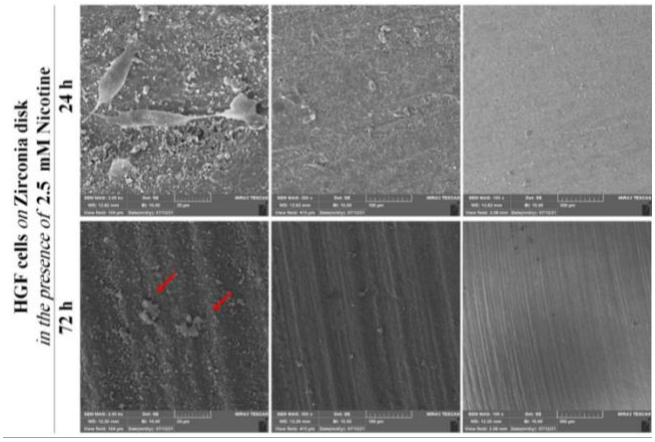

Figure 9. SEM images (20, 100, and 500 µm) of attached HGF cells zirconia surface, 24 and 72 h after exposure to a 2.5 mM concentration of nicotine; red arrows point at the apoptotic cells.

4. Discussion

Long-term implant survival is not only dependent on achieving proper osteointegration but also relies on the quality of the soft tissue seal around the implant. A suitable seal is formed between the peri-implant epithelium and connective tissue, as well as between the implant abutment and the surrounding tissue, providing this seal. This contact is influenced by the health state of the gingival tissue, as well as the material and surface characteristics of the implant components (24-26). Titanium and zirconia implants have been widely used in recent years. This study aimed to evaluate and compare the proliferation, viability, and adhesion of HGFs to titanium, zirconia, and polystyrene surfaces in the presence of various nicotine concentrations.

The findings of the current study indicated no difference between the titanium and zirconia groups in terms of surface roughness. Initial adhesion of vital HGFs (at 24 hours of evaluation) in the presence of high concentrations of nicotine (2.5 and 5 mM) was higher in the titanium and zirconia groups than in the polystyrene group. After 72 hours, the number of vital cells attached to all surfaces was low, with no significant difference between the groups. Surface characteristics such as substrate topography, surface diffusion, and surface energy impact cell-surface affinity, cell migration, and cell spreading (14,27). A previous evaluation of the cellular viability and proliferation between titanium and zirconia with rough and machined surfaces revealed no difference between the materials; however, the cells' morphology was significantly different between rough and machined surfaces in both materials. As a result, it was stated that, rather than the material, surface roughness could influence the biological

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behaviors of the HGFs (28). Comparable surface roughness between the zirconia and titanium specimens might be the reason for similar but higher initial cell adhesion and viability compared to polystyrene surfaces.

According to Esfahanizadeh et al. (8), cell viability and proliferation were higher on Laser-Lock surfaces compared to those on zirconia and titanium groups. Most cells were elongated, spindle-shaped, with pseudopod-like processes, indicating a higher surface affinity. A similar portion of round and spindle-shaped cells was observed in the titanium samples, whereas in the zirconia groups, most cells were round. The viability and proliferation of cells in zirconia and titanium were similar, which was in line with the current study. Furuhashi et al. reported that titanium and zirconia both exhibited similar but superior cell adhesive characteristics compared to the platinum-gold alloy (29).

Another *in vitro* study reported that non-aged zirconia had similar HGF viability compared to titanium. SEM evaluations revealed flattened and elongated phenotypes on titanium and non-aged zirconia, respectively. However, it was noted that the ageing of zirconia significantly reduced the cells' metabolic activity and viability, but it did not alter the zirconia surface roughness or cell attachment features (30). As reported by Kim et al., although the rate of cell proliferation was not significantly different between the zirconia and titanium groups for up to 72 hours, the proliferation rate in the zirconia samples was significantly higher after 7 days of incubation (10). Moreover, the titanium and zirconia groups showed similar results in terms of cell attachment, with both exhibiting higher cell adhesion than the other experimental groups (chrome-cobalt-molybdenum alloy, titanium nitride-coated, anodised titanium, composite resin-coated titanium, and polystyrene culture plates, which served as the control group).

Nothdurft et al. used the fluorescence imaging technique to evaluate HGF-1 and epithelial cell growth on titanium and zirconia surfaces that were machined, smoothed (polished), and roughened (sandblasted) after 24 and 72 hours of incubation (9). In contrast to our findings, they discovered significantly higher HGF proliferation in the zirconia group compared to the titanium group, despite having comparable topography. Among zirconia groups, rough surfaces and, in the titanium groups, smooth surfaces resulted in the highest cell numbers. On the other hand, MTT and SEM evaluations from days 1–21 in the Jung et al. (31) study showed that cell growth and proliferation in the zirconia groups were less than those in the titanium groups, and surface roughness did not affect the cells' biological behaviours. Variations in substrate chemistry and surface topography may be the reason for different results.

Our results indicated that high concentrations of nicotine adversely affected the HGFs' cell adhesion and reduced the proliferation of the attached cells, regardless of the surface material. In addition, an increase in the cells' exposure time significantly reduced their adhesion and viability (time and concentration-dependent cytotoxicity). SEM images exhibited more apoptotic cells in samples with higher nicotine concentrations (2.5 and 5 mM). According to the inverted microscope evaluation, along with the increase in nicotine concentrations, the number of elongated spindle-shaped cells decreased, while more cells showed round, small apoptotic phenotypes. This may explain the lower cell-surface affinity observed at high concentrations of nicotine. These results were in agreement with the findings of Esfahrood et al. and Alanazi et al. (18, 32).

Rouabhia et al. investigated the effect of conventional and electronic cigarette smoke (nicotine-rich with 18 mg of nicotine and nicotine-free) on the adhesion of osteoblasts to titanium surfaces. The cells were exposed to conventional or electronic vapors for 15–30 minutes once daily for 1, 2, or 3 consecutive days. It was found that nicotine concentration and the number of days of nicotine exposure were significantly effective in decreasing adhesion proteins (e.g., F-actin) and reducing alkaline phosphatase (ALP) activity, thereby reducing cell adhesion and growth. Additionally, there was an increase in the caspase-3 protein level, which is a key mediator of cell apoptosis (33).

According to Checchi et al., high concentrations of nicotine (600 mg) can adversely affect the function of HGFs, including reduced cell growth and adhesion (34). This can explain the higher incidence and severity of periodontal disease in heavy smokers. Evaluating a small number of samples for surface roughness without considering confounding factors of the oral environment, such as saliva, intraoral bacteria, and zirconia ageing, raises questions about the generalizability of the results. It is recommended to conduct the evaluations in an *in vivo* setting, involving other cell types, such as mesenchymal stem cells, osteoblasts, and epithelial cells, in addition to the HGFs. Increasing the number of experiment reruns and considering more variations in the samples' surface roughness makes the comparison of the final results with the findings of the other studies more applicable.

5. Limitations

Despite the significant findings, this study has several limitations that should be acknowledged. First, as an *in-vitro* model, it cannot fully replicate the complex oral environment, including saliva, the microbiome, and host immune responses. Second, continuous nicotine exposure in culture media does not reflect the pulsatile nature of real-world smoking or vaping, nor does it account for the numerous other toxic constituents of tobacco smoke. Third, the study was confined to a single cell line (HGF-1) over a 72-hour period, leaving the long-term effects of nicotine on other peri-implant cells - such as osteoblasts and epithelial cells - unexplored. Finally, the relatively smooth surfaces of the titanium and zirconia discs used ($Ra < 0.5$ μm) may not represent the rougher, modified surfaces of contemporary dental implants, which could interact differently with fibroblasts under nicotine-induced stress. Future in vivo and longitudinal studies are needed to validate and extend these findings.

6. Conclusion

An increase in nicotine concentration or the timing of nicotine exposure had an adverse effect on the viability, proliferation,

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and cell-surface affinity of human gingival fibroblasts in all experimental groups. Under the conditions of this *in vitro* study and with comparable surface roughness, no significant superiority of either material was observed. The effect of nicotine cytotoxicity on fibroblasts was independent of the surface material.

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Ethics
This study was approved by the ethics committee of School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.DRC.REC.1400.084).

Using artificial intelligence (AI)
It is declared by the authors of this manuscript that no generative artificial intelligence (AI) or AI-assisted technologies were used to generate content, ideas, or theories during the writing process of this work.

Author contribution
Hosna Kazemi: Investigation, Writing - Original draft
Maryam Torshabi: Conceptualization, Methodology, Data curation, Validation, Resources, Writing - Review & editing
Amirhossein Zamanian: Conceptualization, Supervision, Writing - Review & editing
Zeinab Rezaei Esfahrood: Conceptualization, Writing - Review & editing

Conflict of interest
The authors declare no conflict of interest.

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