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ORIGINAL RESEARCH

Evaluation of Adhesion and Viability of Human Gingival Fibroblasts on Strontium-Coated Titanium Surfaces: an in vitro Study

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Background: Applying multifunctional coatings employing strontium (Sr) ions on titanium (Ti) surfaces is a useful and biocompatible method to improve osseointegration and prevent tissue infections through antimicrobial activity. Nonetheless, the effectiveness of Sr coating on the adhesion and viability of human gingival fibroblasts (HGFs) to Ti surfaces remains unclear.

Purpose: The study aimed to evaluate the effect of Sr coating on the adhesion and viability of HGFs to Ti surfaces.

Materials and Methods: The Ti wafers were divided into two groups based on Sr coating: uncoated Ti (control) and Sr-coated Ti. The Magnetron sputtering technique was used for Sr coating on Ti surfaces. The HGFs were seeded onto the surfaces and cultured for 48 and 96 hours before the cell adhesion and viability of the attached HGFs were assessed. The adhesion of HGFs was analyzed using the attached cell numbers at 48 h and 96 h, and the morphology at 24 h and 72 h. The cytotoxic effect on HGFs was assessed after 24 and 72 hours of incubation using cell viability assay. Student's *t*-test was used for statistical analysis.

Results: The number of cells attached to Sr-coated surfaces was significantly greater than those attached to uncoated Ti surfaces after 48 hours ($P<0.0001$) and 96 hours ($P=0.0002$). Sr-coated and uncoated Ti surfaces were not cytotoxic to HGFs, with the cell viability ranging from 92% to 105% of the untreated control HGFs. There were no significant differences in cell viability between Sr-coated and uncoated Ti surfaces at 24 hours ($P=0.3675$) and 72 hours ($P=0.0982$).

Conclusion: Sr-coated Ti surfaces induce adhesion of HGFs compared to uncoated Ti surfaces. Further, Sr-coated and uncoated Ti surfaces show no cytotoxic effect on the attached HGFs.

Keywords: cytotoxicity, dental implant, peri-implantitis, metal ions, multifunctional coatings, titanium surfaces

Introduction

Tooth loss, often due to caries, periodontal diseases, or trauma, is a major global public health concern that affects adults even at an early age, leading to impaired oral health-related quality of life.[1] Consequently, there is an increased interest and demand for replacement of missing teeth to restore function and esthetic appearance. Currently, dental implants made of titanium (Ti) are one of the most reliable treatment modalities for replacing missing teeth with high success and survival rates.[2,3] Owing to the biocompatibility and the great load-bearing capacity of Ti, the reported survival rates of dental implants increased up to 95% with 10 years of function.[4] Nonetheless, other material factors should be considered when striving to achieve a successful implant treatment, eg, surface chemistry and surface macro- and micro-topography.[5]

Indeed, surface characteristics play a significant role in the osseointegration between the surrounding bone tissue and the surface of the implant fixture; hence, significant research has been conducted to improve bone-implant contact (BIC).[6,7] However, those surface characteristics can influence cell adhesion and viability, affecting the attachment of peri-implant connective tissues on implant surfaces, thus, jeopardizing the treatment outcome.[8,9] Based on earlier

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research, the connective tissue barrier around implant surfaces, which is rich in fibroblasts, is a crucial factor for protection against bacterial invasion that can lead to peri-implantitis.[10,11] The latter has been the subject of thorough investigation, identifying plaque and biofilm contamination as the leading causes of peri-implantitis.[12,13] Additionally, tissue level implant, in particularly, has a machined surface that penetrates the connective tissue, to be connected with the abutment and superstructure, providing direct communication and adhesion with fibroblast.

Several implant surface treatments have been developed including roughening the implant surface for better mechanical interlocking with surrounding peri-implant tissues.[5] Even though a rough surface increases BIC and fibroblast cell adhesion, it is associated with a higher risk of peri-implant infections due to bacterial attachment and biofilm formation.[14,15] Other strategies involving multifunctional coatings on Ti implant surfaces, eg, antibiotics, antimicrobial peptides, and polysaccharides have been proposed for peri-implant infection prevention.[16–18] However, there are some concerns regarding using such strategies, eg, cytotoxicity, coating stability, and bacterial resistance, further accentuating the need for the development of multifunctional coatings improving the osseointegration and peri-implant soft tissue adhesion, while preventing peri-implant infections simultaneously.

Surface modification of Ti implants with metal ions, ie, strontium (Sr), has shown a remarkable ability to improve osseointegration and maintain excellent biocompatibility outcomes.[19,20] Moreover, recent studies have demonstrated the potential antimicrobial properties of Sr against multiple bacteria associated with peri-implantitis.[21,22] Nevertheless, the effect of modifying Ti surfaces with Sr metal ions on the adhesion and viability of human gingival fibroblasts (HGFs) is still unclear. These connective tissue cells are responsible for growing efficient soft tissues around the implant.[10,11] They thereby prevent the accumulation of submucosal bacterial plaque and contamination of implant surfaces. Therefore, the present study evaluated the effect of Sr coating on the adhesion and viability of HGFs to Ti surfaces. The null hypothesis of this study was that Sr coating does not improve the adhesion and viability of gingival fibroblasts to Ti surfaces.

Materials and Methods

Sample Preparation and Surface Treatment

Commercially pure Ti wafers coated with Sr, using magnetron sputtering technique, and uncoated Ti wafers were obtained from ELOS Medtech® (Gorlose, Denmark). The wafers underwent a sputter-cleaning process for 10 minutes with a bias of 800 V, duty factor of 30%, and working pressure of 0.02 Pa. Then, they were sputtered with Ti for 10 minutes with a bias of 250 V, duty factor of 30%, and working pressure of 0.02 Pa. After that, Sr deposition was carried out by sputtering at a substrate bias of 150 V, duty factor of 80%, and working pressure of 0.02 Pa for 40 minutes. The Sr-coated and uncoated Ti wafers were initially cut into small square-shaped samples ca. 5 × 5 mm and then disinfected using 70% alcohol for 5 minutes according to manufacturer's instructions. Following disinfection, the samples were washed twice using phosphate-buffered saline (PBS) (PAN-Biotech GmbH, Aidenbach, Germany) and then transferred to 24-well plates (Ibidi® µ-Slide, Ibidi GmbH, Martinsried, Germany) for testing.

Isolation of Fibroblasts and Cell Culture

This study complies with the Declaration of Helsinki and was performed according to ethics committee approval by Umm Al Qura University (approval number XDYS170124). The gingival tissues were collected from a healthy adult dental patient undergoing crown lengthening surgery at Umm Al Qura Dental Teaching Hospital after obtaining written informed consent. The gingival tissues were washed with PBS (PAN-Biotech GmbH, Aidenbach, Germany). A 1 mg/mL of dispase® (Sigma-Aldrich, St. Louis, MO, USA) was added to the gingival tissues before eight-hour incubation at 4°C. After removal of the epithelial layer, the connective tissue was cut into small pieces and cultured in a complete cell growth medium in a 25 mL tissue culture flask. The gingival fibroblasts were cultured and grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone™, Thermo Fisher Scientific, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), and 2.5 µg/mL amphotericin B (Gibco) at 37°C and 5% CO2 in a humidified atmosphere incubator. The culture medium was changed every 2–3 days. Sufficient fibroblast proliferation was observed in passage four, thus gingival fibroblasts were frozen (−150°C) at passage four and stored until use.

Fibroblast Adhesion

Duplicate samples of Sr-coated and uncoated Ti were transferred to 24-well plates using sterile tweezers and placed in the well, with the test surface facing upward. Afterwards, fibroblasts were seeded at 1×10^5 per well on the surfaces of Sr-coated and uncoated Ti samples and cultured for 48 and 96 hours. During this incubation period, viable fibroblasts attach to the titanium surface. After each time-point, each sample with attached fibroblasts was transferred to a new well of 24-well plate to assess the cell viability of the attached fibroblasts and exclude the cells attached to the 24-well plate surface. The cell viability, which reflects the cell number attached to the titanium surface, was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Thermo Fisher Scientific, USA) which is a colorimetric assay based on cell metabolic activity.

Cell Viability (Cytotoxicity) and MTT Assay

To assess the cytotoxic effect of Sr on the gingival fibroblasts, the cells were seeded at 5 × 104 per well in a 24-well plate and incubated in DMEM at 37°C in a humidified atmosphere of 5% CO_2. After 24 h of incubation, duplicate samples of Ti were placed on the fibroblast monolayer. Following 24 h and 72 h of incubation, the plates were examined under an inverted microscope (Eclipse TS 100, Nikon Instruments Inc., Tokyo, Japan), and the cell viability MTT assay was performed. The medium was removed and replaced with 500 µL per well of culture medium containing 0.5 mg/mL of MTT and incubated for 3 hours at 37°C. Afterwards, the medium was removed, and 400 µL per well of dimethyl sulfoxide solvent solution (DMSO) (Sigma-Aldrich) was added to dissolve the formazan crystals. Triplicate of 100 µL per well of the resulting purple solution were transported to a 96-well plate, and the optical density was measured at 570 nm on a Spectrophotometric Microplate Reader (SpectroStar® Nano, BMG Labtech, Ortenberg, Germany) to calculate the cell viability relative to untreated control fibroblasts.

Statistical Analysis

GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel were used for data collection, statistical analysis, and graphs. The results are expressed as mean ± standard error of the mean (SEM) and analyzed using unpaired *t*-tests. A p-value ≤ 0.05 was considered significant.

Results

Morphological Changes in Human Gingival Fibroblasts

The fibroblasts treated with either Sr-coated or uncoated Ti samples were examined after 24 and 72 h by a light inverted microscope at 100X magnification. The untreated control fibroblasts appear as fusiform, spindle-shaped adherent cells growing as a confluent monolayer. No changes were observed in the morphology of the fibroblasts treated with Sr-coated or uncoated Ti samples compared to untreated control fibroblasts (Figure 1).

Viability of Human Gingival Fibroblasts

The viability of the gingival fibroblasts grown on Sr-coated and uncoated Ti surfaces was determined after 24 h and 72 h by the MTT assay. Both Sr-coated and uncoated Ti surfaces were not cytotoxic to the gingival fibroblasts, with the cell viability ranging from 92% to 105% of the untreated control cells (Figure 2). There were no significant differences ($p > 0.05$) in cell viability between Sr-coated and uncoated Ti surfaces at 24 hours ($P = 0.3675$) and 72 hours ($P = 0.0982$).

Human Gingival Fibroblast Attachment

The viability of gingival fibroblasts attached to the tested surfaces was assessed by MTT assay after 48 and 96 hours of incubation. The number of cells attached to Sr-coated Ti surfaces was significantly greater than those attached to uncoated Ti after 48 hours ($p<0.0001$) and 96 hours ($p=0.0002$) (Figure 3).

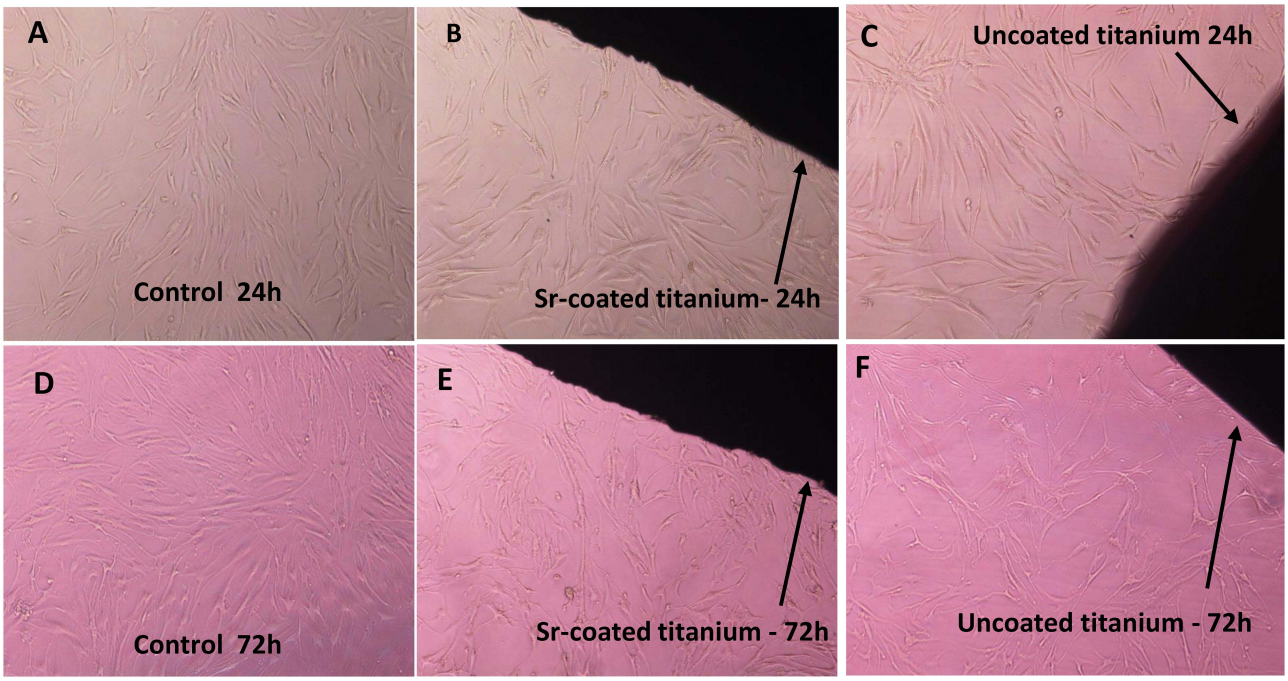

Figure 1 Morphological changes in gingival fibroblasts cultured in Sr-coated and uncoated Ti samples. The cells were examined after 24 (**A-C**) and 72 hours (**D-F**) by an inverted microscope at 100X magnification.

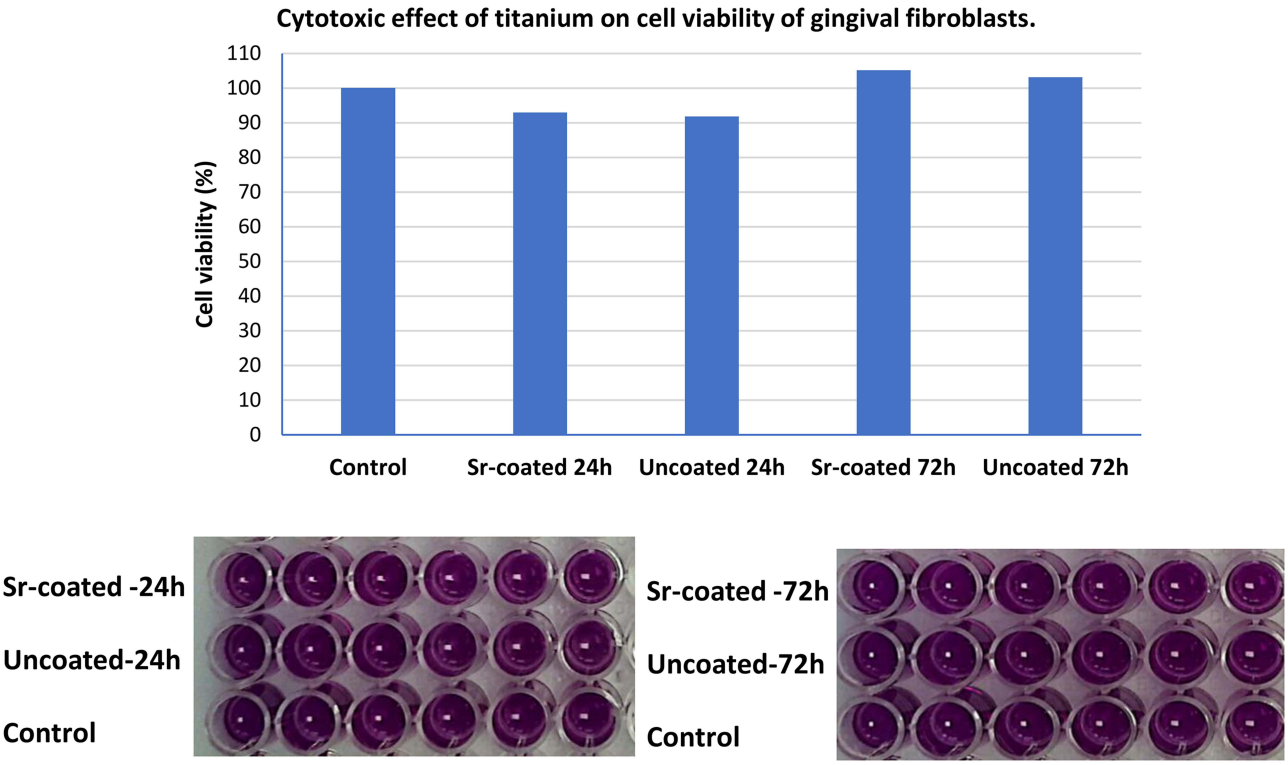

Figure 2 Cell viability of gingival fibroblasts in the presence of Ti. The gingival fibroblasts were grown on Sr-coated and uncoated Ti surfaces and the cell viability was determined after 24 hours and 72 hours by MTT assay. The untreated control cells represent 100%.

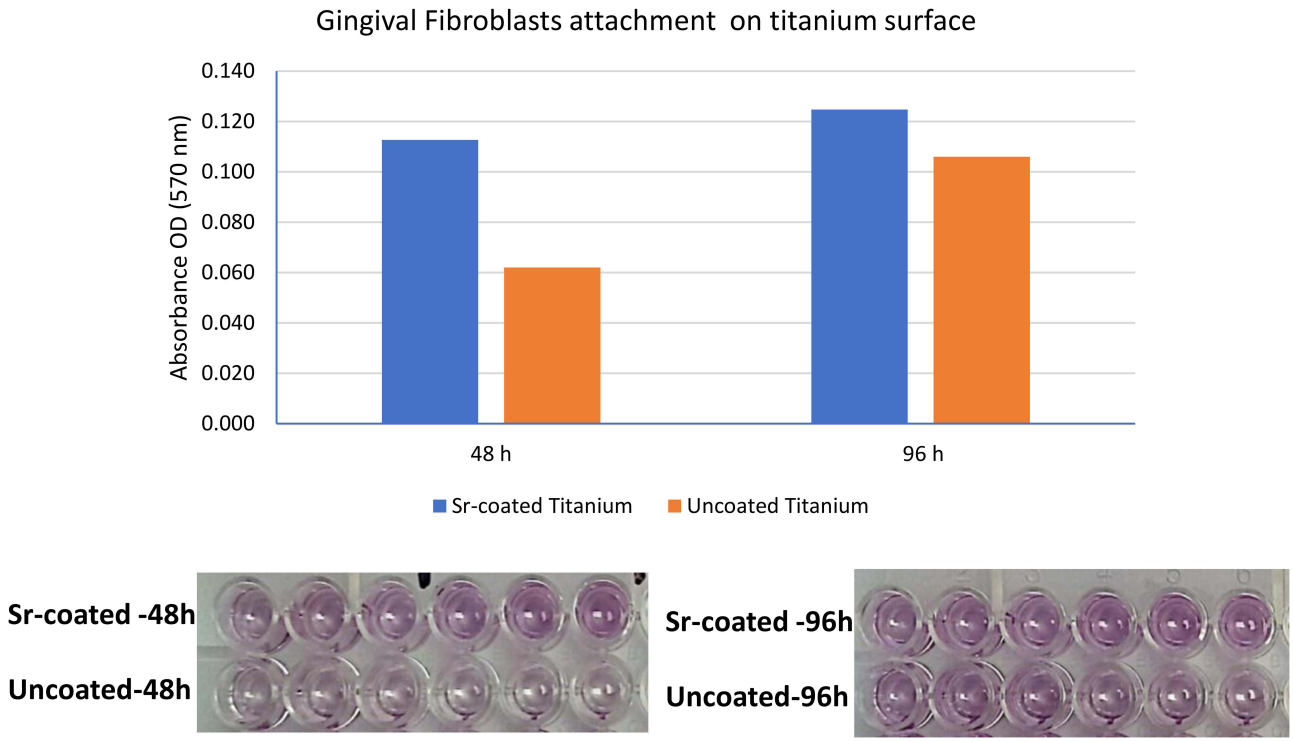

Figure 3 Gingival fibroblast attachment to Sr-coated and uncoated Ti surfaces after 48 hours and 96 hours of incubation.

Discussion

The current study aimed to evaluate the ability of Sr coating on Ti surfaces to promote HGFs adhesion and viability. The main findings revealed that Sr coating on Ti surfaces can promote HGFs adhesion without reducing viability; hence, the null hypothesis of the study was rejected. The number of viable cells attached to Sr-coated Ti surfaces was significantly greater than those attached to uncoated Ti surfaces after 48 hours and 96 hours. Additionally, the study found that both Sr-coated and uncoated Ti surfaces were not cytotoxic to HGFs, with the cell viability ranging from 92% to 105% of the untreated control HGFs.

Titanium is a commonly used material for dental implants. Modifying the Ti surface by applying multifunctional coatings with antimicrobial potential and efficient osseointegration capacity is common in dental implantology to ensure successful implant treatment. However, there is a lack of research regarding the effect of multifunctional coatings on fibroblast adhesion and growth proliferation of the connective tissues on the transmucosal part of the dental implant. In this study, we aimed to evaluate whether treating Ti surfaces with Sr-functionalized coating leads to a surface attractive to HGFs.

Surface modification of the Ti implant using Sr-containing coatings has been investigated in previous studies, resulting in enhanced BIC capacity with antimicrobial properties against the growth of several bacterial species associated with peri-implantitis.[19–22] Sr ions are considered safe compared to silver (Ag) ions that exhibit high cytotoxicity and there are concerns related to systemic toxic issues.[23,24] However, Sr-containing coatings might have an influence on the adhesion and growth proliferation of HGFs on Ti surfaces.

A comparison of Sr-coated and uncoated Ti surfaces revealed that more HGFs were attached to Sr-coated Ti surfaces. Furthermore, the adhered cells were viable and able to proliferate, and neither the Sr-coated nor uncoated Ti surfaces were cytotoxic to HGFs. These results agreed with previous studies that show the high biocompatibility of Ti and Sr with low levels of cytotoxicity.[2,19] Furthermore, HGFs seeded onto Sr-coated and uncoated Ti surfaces were not morphologically different from untreated control fibroblast cells, further affirming the biocompatibility of both Sr and Ti. The adherent cells on Sr-coated Ti surfaces exhibited spindle-shaped (fusiform) morphology with cellular bridges that grew as

a confluent monolayer. Previous studies have shown that this cell morphology has a higher ability for attachment than round cells,[25] indicating that the fusiform morphology of adhered fibroblasts in this study might be associated with more cell adhesion and proliferation, and hence could provide better circumstances for successful implant treatment. Moreover, this study determined the quantity of live cells present in Sr-coated Ti surfaces by assessing their cell viability. Cell viability was evaluated using assays that measure specific features unique to living cells, such as membrane integrity and metabolic activity.[26] The results of these tests were essential to understanding the impact that Sr-coated Ti surfaces have on living cells. An increase in cell viability suggested that the surface coating has a protective or stimulatory effect on the cells. In contrast, a decrease in viability indicated that the surface coating may have a harmful effect. Therefore, the findings of fibroblast adhesion on Sr-coated Ti surfaces in this study can help to design and develop safer and more effective implants.

Several studies have been conducted to explore the effectiveness of using a variety of metals, instead of Sr, as multifunctional coatings on Ti surfaces.[9,23,24] One such metal is tantalum (Ta), which has shown remarkable biocompatibility, corrosion resistance, and antibacterial action when applied as a coating on Ti surfaces.[27–29] Moreover, in a similar study, it was found that Ta coating promotes the adhesion, viability, and proliferation of HGFs on Ti surfaces that makes it highly suitable for use in the transmucosal portion of dental implants.[9] Additionally, combining Sr with Ta in hydroxyapatite coating material has been shown to result in excellent biocompatibility with osteoblast-like cells, leading to enhanced bone growth on titanium surfaces.[30] Given the excellent proliferation of osteoblast-like cells in those hybrid coating layers, modifying the titanium surface using a combination of Sr and Ta in future studies might provide better adhesion behavior of HGFs compared to using Sr alone. This could have significant implications for the field of dental implantology, as it could lead to the development of more effective coatings that promote better osseointegration with enhanced attachment of peri-implant soft tissues, reducing the risk of implant failure.

Besides the inherent characteristics of Sr ions, the surface properties might also play a significant role in gingival fibroblast adhesion and proliferation in the present study. Cells have been shown to have different adhesion and proliferation capacities depending on the surface topography. Rough surfaces enable more cell adhesion and proliferation compared to smooth ones, but they also allow undesirable bacterial adhesion and biofilm formation.[14,15] Previous studies demonstrated that increasing the roughness of implant components in contact with soft tissues might cause peri-implant tissue inflammation.[14,15,31] Moreover, gingival fibroblasts on smooth polished surfaces were spread without specific orientation, leading to poorer adhesion and proliferation.[32] In this study, Sr-coated wafers have shown increased cell attachment and viability and, thus, could possibly provide a solution for low cell attachment to the smooth surfaces. The present study utilized magnetron sputtering technique to obtain a uniform Sr film deposited on the Ti surface. Magnetron sputtering is a superior physical vapor deposition technique offering high deposition rates, high-purity films, and strong adhesion of multifunctional coatings on Ti substrates.[33] Further, magnetron sputtering technique could provide similar uniform coatings on complicated irregular surfaces, eg, dental implant. All wafers tested were produced by the manufacturer in a standardized way to ensure the same surface topography. Nonetheless, one of the study limitations was that Sr-coated and uncoated surfaces were not characterized besides the adhesion and viability of fibroblasts. Despite this study not investigating the surface characteristics, the improved adhesion of fibroblasts might be related to the altered implant surface roughness acquired by the magnetron sputtering technique in addition to characteristics of Sr metal ions. However, since the different smoothness of Ti surfaces can be determinantal for peri-implantitis and successful implant treatment, further studies are required to investigate the surface topography of Sr-containing coatings on Ti surfaces.

Although the study found that Sr-coated Ti surfaces had superior cell attachment, it is essential to note that this was an in vitro investigation, and the conditions tested may not completely represent the complex environment of the oral cavity. Furthermore, the study only evaluated one aspect of the complex environment between the Ti surface and peri-implant tissues, in particular, the adhesion behavior and viability of fibroblast cells on Ti surfaces, with and without Sr coating. Other important aspects of this complex environment, for instance, evaluating the growth and adhesion of other mucosal cells on Ti surfaces and the effect of loading conditions to detach the adhered cells on the implant surface should be considered in future research. Nonetheless, the ease of conducting the experiments in this study allows consistent settings, a uniform analysis of different cells from the oral mucosa, and, as a result, comparable findings.

Conclusion

Within the limitations of this in vitro experimental study, this work shows that Sr-coated Ti surfaces induce the adhesion of HGFs compared to uncoated Ti surfaces. Further, Sr-coated and uncoated Ti surfaces show no cytotoxic effect on the attached HGFs. However, further in vivo studies are required to explore the clinical application potential of Sr ions.

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Disclosure

The authors report no conflicts of interest in this work.

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