Influence of Titanium Surface Treatments on Viability of Periodontal Fibroblasts Grown in an Osteogenic Culture Medium

Abstract

Background: The integrity of the protective seal provided by the gingiva in direct contact with the implant surface is one of the main factors involved in the prevention of peri-implantitis. Aim: The aim of this study was to assess the viability of periodontal fibroblasts grown in an osteogenic culture medium in contact with titanium surfaces treated either with acid etching alone or with acid etching + anodizing. Materials and Methods: Periodontal fibroblasts grown in an osteogenic culture medium were distributed in a control group, with cells grown in culture bottles, and two experimental groups, with cells grown in contact with titanium disks measuring 6 mm in diameter. The surface of the disks was subjected to acid etching alone (AEG, n = 25) or to acid etching + anodizing (ANG, n = 25), and then evaluated using scanning electron microscopy (SEM). Cell viability was assessed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] bromide test on days 1, 2, 3, 7, and 14 of the cell culture. The Mann–Whitney test was used for the statistical analysis (P < 0.05). Results: The SEM assessment revealed that the surface of AEG specimens had micrometric characteristics, whereas the surface of ANG specimens had nanometric characteristics. No significant difference was observed among the groups regarding cell viability at any of the evaluation time points. Conclusion: The titanium surface treatments tested did not affect the viability of periodontal fibroblasts in an osteogenic culture medium.

Keywords: Biocompatible coated materials, culture media, fibroblasts, scanning electron microscopy, titanium

Introduction

Installation of transcutaneous implants such as dental implants, cochlear hearing devices, and other prostheses, can cause infections or various tissue changes caused by improper closure of the interface between implant biomaterial and soft tissue.[1] The skin or gingiva in direct contact with the implant surface provides a protective seal between the peri-implant bone and the external environment, and the integrity of this seal is one of the main factors involved in the prevention of infection.[2] The viability and differentiation of cells in contact with the surface of new implant materials are commonly investigated in vitro to ascertain the potential of these materials to promote cell adhesion.[3,4] Cell viability tests can be used to assess this characteristic on titanium (Ti) surfaces, the main metallic component of dental implants.[5] These tests have shown that gingival and/or periodontal fibroblasts can adhere to the Ti surface of the cervical portion of the implant, or, in some cases, to the surface of the prosthetic components installed on the implant, thereby establishing an interface between the implant and gingival tissue.[6]

However, the force of cell adhesion to Ti may not be sufficient to ensure the integrity of this interface, and new Ti alloy compositions and surface modifications have been developed to increase cell adhesion.[7] It has already been established that the physical characteristics of a Ti surface are critical for successful dental implant treatment and for the long-term health of peri-implant tissues.[8] The stromal cells of gingival tissue display very poor attachment to metallic surfaces and their direct contact with metallic biomaterials may lead to inflammation, infection, and decreased cell viability.[9–11] To this end, changes in implant surface roughness, topography, and chemical composition have been investigated to increase the viability of cells in contact with this surface.

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Another factor that can affect cell viability on the metallic surfaces of implants is the mediators of osteogenic differentiation, observed throughout the osteointegration phase. These mediators can reduce the ability of cells to proliferate by pushing them to differentiate.\[^{12}\] Thus, the aim of this study was to evaluate the viability of periodontal fibroblasts grown in an osteogenesis-inducing culture medium in contact with Ti disk surfaces treated either with acid etching or anodizing techniques.

**Materials and Methods**

This study was approved by the research ethics committee of the institution where it was conducted (Approval no. 2017/0774).

**Cell culture**

Human periodontal ligament fibroblasts were grown in Dulbecco’s Modified Eagle Culture Medium (DMEM; Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (Cultilab) and 1% of an antibiotic-antimycotic solution (Sigma; St. Louis, MO, USA). The cell culture bottles were kept in an incubator at 37ºC under a humid atmosphere containing 95% oxygen and 5% carbon dioxide. The culture medium was changed every 48 h until the cells reached a confluency of 80%. The cells were then removed from the bottles and cryopreserved until use in the experiment.

**Titanium disks**

Fifty commercially pure (grade IV) Ti disks, 6 mm in diameter and 2 mm thick (Conexão Sistema de Prótese; Arujá, SP, Brazil), were distributed into two experimental groups according to the assigned surface treatment: Acid etching (AEG, \( n = 25 \)) or acid etching + anodizing (ANG, \( n = 25 \)) [Figure 1]. Initially, the surfaces of all of the disks were sandblasted with 180-µm aluminum oxide particles at a pressure of 0.25 MPa, producing Ra values ranging from 1.5 to 2.5. Next, they were submitted to acid etching for 20 min in a 5 N HNO\(_3\) + 5 N HF solution, at a temperature of 20°C. Half of the specimens were then submitted to an anodizing bath of 1.0 M phosphoric acid at 20°C, with a current density of 5 mA/cm\(^2\) maintained by a stabilized voltage of 80 V. The anodizing process promoted the formation of a 120-µm thick oxide layer within approximately 30 s.

The disk surfaces were evaluated using scanning electron microscopy (SEM), and the microscope (JSM-6460LV; Jeol, Tokyo, Japan) was operated at an acceleration voltage of 20 keV. The SEM images were reconstructed using SMile View Map software (Digital Surf, Besançon, France; Jeol, Peabody, MA, USA). Representative images of scanned disk areas are shown in Figure 2. All of the images were acquired with a resolution of 1280 × 960 pixels.

**Osteogenic culture medium and cell viability test**

After left to thaw, the cells were grown in 75-mL bottles for 14 days in an osteogenic culture medium consisting of DMEM (Cultilab) with a high glucose concentration (4.5 g/L), L-glutamine (584 mg/L; Cultilab), sodium pyruvate (110 mg/L; Cultilab), 20% iron-supplemented fetal bovine serum (Cultilab), penicillin (100 IU/mL; Sigma), and streptomycin (100 µg/mL; Sigma). The medium was buffered with sodium bicarbonate (1 N; Sigma). The pH was adjusted to 7.2, and 0.5 µg/mL of ascorbic acid (Sigma), 10 mmol/L of β-glycerophosphate (Sigma), and 10 mmol/L of dexamethasone (Sigma) were added to the solution. The cells were kept in an incubator at 37°C under a humid atmosphere containing 95% oxygen and 5% carbon dioxide. The culture medium was changed every 48 h.

Cells kept in the bottles formed the control group (CG); of these, 2.5 × 10\(^4\) cells were then seeded onto the Ti disks described above, thus forming experimental

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**Figure 1:** Titanium disks used in the study. (a) Surface treated with acid etching alone; (b) Surface treated with acid etching + anodizing (this figure is original)

**Figure 2:** Scanning electron microscopy images of the titanium disks. (a) Surface treated with acid etching alone, at × 50,000; (b) surface treated with acid etching alone, at × 150,000; (c) surface treated with acid etching + anodizing, at × 50,000; (d) surface treated with acid etching + anodizing, at × 150,000 (this figure is original)
groups AEG (acid etching alone) and ANG (acid etching + anodizing). Cell viability was assessed on days 1, 2, 3, 7, and 14 of cell culture (five disks per assessment time point) using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] bromide test (MTT; MTT Assay Kit ab211091; Abcam, Boston, MA, USA), conducted according to the manufacturer’s instructions. The cell metabolic activity results were read at 570 nm using a spectrophotometer (Epoch Microplate Spectrophotometer; BioTek Instruments, Winooski, VT, USA) and expressed in arbitrary absorbance units, where the greater the signal emitted, the greater the metabolic activity of the analyzed cells.

**Statistical analysis**

The data were analyzed using the Mann–Whitney test. *P < 0.05 was considered statistically significant.

**Results**

The SEM assessment revealed that the disk surfaces of the AEG and ANG groups had distinguishable characteristics under both 50,000 and 150,000 magnification. The surfaces treated with acid etching alone displayed a shallow, irregular, and micrometric relief, whereas those treated with acid etching + anodizing displayed a tubular and nanometric relief. Table 1 presents the values of the readings of metabolic activity of the periodontal fibroblast cultures in the three study groups.

**Discussion**

There was no difference among AEG, ANG, and CG regarding the values of metabolic activity at any of the evaluation time points; therefore, the null hypothesis was not rejected. These results contrast with those of Kim et al.,[13] who found that the viability of periodontal ligament cells gradually increased over time, and decreased with an increasing concentration of dexamethasone; and also with those of de Vries et al.,[14] who found that both proliferation and viability of fibroblasts in an osteogenic medium increased over time in three-dimensional cultures.

The chemical, mechanical and topographic characteristics of implant surfaces can affect the adhesion of cells involved in bone formation at the bone-implant interface. In addition, these characteristics can favor cell proliferation and differentiation, and stimulate cells to deposit osteoid matrix as well. Surface treatments have been used to increase the bone-to-implant contact area, and to shorten the healing time before loading.[15,16] Several Ti surface treatments have been studied in the past decades with the goal of altering the postoperative time required for osseointegration. The most commonly used are those performed with acid etching and acid etching followed by sandblasting.[17]

It has already been established that a treated Ti surface can increase the deposition of fibrin matrix, and thus lead to the formation of thicker blood clots than those produced in contact with a machined Ti surface.[18] Other authors have reported that platelets adhere significantly more effectively to treated Ti surfaces than to machined ones. This effect is particularly important for the immune response and for wound healing, since platelets secrete a multitude of factors involved in the activation of these processes, including platelet-derived growth factor, transforming growth factor beta, and vascular endothelial growth factor.[19–21] The presence of these growth factors is associated with the promotion of adhesion, dissemination, and migration of gingival and periodontal fibroblasts. This observation suggests the existence of a synergistic mechanism between blood and peripheral fibroblasts, which contributes to promoting faster tissue regeneration in contact with treated Ti surfaces.[22]

The present study analyzed the relationship between cells and Ti surface using a cell viability test under conditions of osteogenic differentiation,[23,24] since osteogenic stimuli are the most abundant in the biological microenvironment immediately around dental implants, and in contact with the blood clot, especially in the early stages of regeneration. Furthermore, the Ti surface treatments performed by acid etching alone or acid etching + anodizing produced micro- and nanometric structures, respectively, and were used in the present study because they are among the currently available commercial surface structure modifications.[25,26] Both types of surface treatments have been associated with increased cell adhesion and differentiation,[26] however, previous studies[25,24,26] have used osteoprogenitor cells or osteoblasts, whereas the present study used periodontal fibroblasts to investigate which of these surfaces would better favor the adhesion and proliferation of fibroblasts after their migration. This choice is particularly relevant considering the soft-tissue bond requirement, which plays the role of a biological seal around the dental implant. To this end, an assessment of

| Table 1: Mean and standard deviation values of the readings of metabolic activity of periodontal fibroblasts grown in bottles, in the control group, and in contact with titanium disks, in the experimental groups, measured by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] bromide test assay at a wavelength of 570 nm |
|---|---|---|---|---|---|---|---|---|
| 1 day | 2 days | 3 days | 7 days | 14 days | *P*
| CG | 1.36±0.25 | 2.73±0.16 | 3.07±0.23 | 1.77±0.22 | 1.99±0.22 | 0.3 |
| AEG | 1.99±0.11 | 1.99±0.29 | 2.99±0.40 | 1.73±0.34 | 2.03±0.45 | 0.3 |
| ANG | 2.18±0.35 | 1.51±0.27 | 3.29±0.27 | 1.80±0.17 | 1.83±0.40 | 0.4 |

Values are expressed in arbitrary absorbance units. *Mann-Whitney test (P<0.05). CG: Control group (only culture medium, no disks); AEG: Culture medium applied onto titanium disk surfaces treated with acid etching alone; ANG: Culture medium applied onto titanium disk surfaces treated with acid etching+anodizing.
the ability of periodontal fibroblasts to adhere to modified Ti surfaces can be a useful input in the decision-making process faced by clinicians when choosing the appropriate implant for each treatment plan.

**Conclusion**

The Ti surface treatments tested, either with acid etching alone or with acid etching + anodizing, had no effect on the viability of periodontal fibroblasts grown in an osteogenic medium.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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