Fibroblast response to initial attachment and proliferation on titanium and zirconium surfaces.

Abstract: Introduction: In recent decades, dental implants have become one of the best options for comprehensive dental restoration; their placement is a multidisciplinary task that requires a solid understanding of biological, periodontal, surgical and prosthetic principles. Objective: The aim of this study was to quantify in vitro the adhesion and proliferation of human gingival fibroblasts (HGF) response on titanium (Ti) and zirconia (Zr) surfaces. Methodology: Samples of Ti and Zr were observed under atomic force microscopy (AFM). HGFs were inoculated in each sample to determine adhesion and cell proliferation. The reagent MTT was mixed with medium DMEM and inoculated in each plate; formazan was dissolved with dimethyl sulfoxide and analyzed at 540nm in a microplate spectrophotometer. The test was performed with three independent experiments. Data were analyzed with Kolmogorov-Smirnov tests (Lilliefors), Kruskal-Wallis tests and Mann-Whitney test comparisons. Results: Topography of the Zr plates showed greater roughness (Ra= 0.39μm) than Ti (Ra= 0.049μm). Quantification of HGF adhesion was significantly higher (p<0.05) in Ti, while proliferation showed no statistically significant differences between the groups. Conclusion: It is noteworthy that, even though Ti initially showed increased cell adhesion on the surface, after 24h Zr samples showed similar proliferation; this demonstrates that both surfaces have a comparable biological response.

Keywords: Dental implants, Titanium, Zirconium, Biomaterials, Human gingival fibroblasts.

DOI: 10.17126/joralres.2016.043.

Cite as: Meza-Rodríguez A, Martínez-Álvarez O, Acosta-Torres L, de la Fuente-Hernández J & García-Contreras R. Fibroblast response to initial attachment and proliferation on titanium and zirconium surfaces. J Oral Res 2016; 5(5): 194-199.

INTRODUCTION.

In recent decades, dental implants have become one of the best options for comprehensive dental restoration in patients with partial or total prosthesis. Dental implants may help patients obtain a complete, healthy, functional and aesthetic dentition1. Implant placement is a complex, multidisciplinary task involving various disciplines and requiring a deep knowledge of periodontics, surgery and prosthetics2.

The successful placement of a dental implant is determined by the environment of the oral cavity and the response of adjacent tissues. The success of an implant is directly related to bone formation around it (osseointegration), in order to direct the forces of mastication to the bone structure3; soft tissue integration to provide a biological seal between the oral cavity and the implant2 and the appropriate biocompatibility of the tissue at systemic and local level3. As soon as an implant is placed in the appropriate site, there is a molecular interaction covering the implant in just nanoseconds. This is primarily influenced by the implant surface3, which also determines the type of coating and rehabilitation procedures4.

Anderson defined the term biocompatibility as the ability of a biomaterial, prosthesis or medical device to perform...
a specific function, with an appropriate host response. The evaluation of biological responses is measured by the magnitude and duration of adverse alterations in homeostatic mechanisms that determine the host response⁵.

Biomaterials commonly used for dental implants are manufactured from metal (titanium and its variants) and ceramic materials covered by porcelain (aluminum and zirconium oxide)⁶. Ti is widely used because it has proven to be a biocompatible and bio-inert material, stable and very well tolerated by soft tissues⁷. Although Ti implants have been used as the gold standard in the past 40 years, cell adhesion to this type of material is not always strong, and new formulations and modifications of the surfaces are developed to enhance cell attachment to the implant and accelerate osseointegration⁸.

In response to the above, the use of dental implants based on Zr represents a new frontier in implantology. Ceramic implants have been successfully used in orthopedic surgeries for many years because their biocompatibility tests have yielded positive results, while carcinogenicity and mutagenicity tests have shown negative results. Zr can provide an aesthetic advantage and result in less biofilm accumulation on the surface of the implant⁹. However, due to the lack of information on their performance in the short and long term¹⁰, Zr implants have not been widely used as Ti ones.

The hypothesis proposed in this study is based on the fact that a Zr surface can provide adhesion and proliferation for human gingival fibroblasts (HGF) equal to or greater than a Ti surface¹¹, and therefore show more scientific evidence on the adhesion of fibroblast to Zr surfaces.

The aim of this study was to quantify in vitro the adhesion and proliferation of HGF response on Ti and Zr surfaces with a fast and reproducible colorimetric method using MTT bromide salt.

**MATERIALS AND METHODS.**

**Materials**

Minimum essential alpha culture medium (α-MEM) (Sigma Aldrich, St. Louis, Missouri, USA), Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA, USA), fetal bovine serum (FBS, Sigma culture Aldrich, St. Louis, Missouri, USA), phosphate-buffered saline (PBS, Sigma Aldrich, St. Louis, Missouri, USA), penicillin G and streptomycin sulfate (Gibco, Carlsbad, CA, USA), type I titanium (99.5% purity) (Tokuriki Melters, Tokyo, Japan) and zirconium (Zirkonzahn, An der Ahr, Gais, Germany). 0.25% trypsin-EDTA 0.025% -2Na (Gibco, Carlsbad, CA, USA), MTT reagent (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich, St. Louis, Missouri, USA), dimethyl sulfoxide (DMSO, JT Baker, Center Valley, PA, USA), 10-cm culture dishes and 96-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**Preparation of Ti samples**

Type 1 Ti plates of 10x10x0.5mm (n=3) were prepared. Samples were placed in epoxy resin and polished automatically at 160-200 rpm (Buehler, Lake Bluff, IL, USA) with water sandpaper of different roughness, # 400, 800, 1000, 1500 and 2000 (Fujistar, Sankyo, Rikagaku, Okegawa, Japan) and diamond suspension from 0.05 to 1μm with a cloth (Chemomet, Buehler, Lake Bluff, IL, USA). Samples were removed from the epoxy resin and washed in ultrasound with distilled water, 99.5% ethanol and 99.5% acetone for 10 minutes and dried at room temperature.

**Preparation of Zr samples**

Zr plates of 10x10x10mm (n=3) were prepared. Samples were sintered in a conventional manner in a furnace at 2822°F for 6 hours and then the temperature was gradually decreased for 3 hours. Then plates were cut, polished and sandblasted. All Ti and Zr plates were reused for each experiment after being re-polished, ultrasonic washed and sterilized.

**Observation of samples by AFM**

Ti and Zr surfaces were evaluated using atomic force microscopy (NANOSURF FlexAFM, Liestal, Switzerland). The surface roughness was estimated based on Ra (arithmetic mean of the absolute values of the coordinates of the points of the roughness profile in relation to the midline within the length measurement) and Rms (the largest partial roughness present on the measuring
Surface) on a surface of 80x80 μm in tapping mode based on ISO 4287:1997: Geometrical Products Specifications (GPS)-Surface texture: Profile method.

Cell culture
The human gingival fibroblasts (HGF) were obtained from gingival tissue biopsy of a third molar of an 18-year-old patient, with prior approval and after signing informed consent. The project was authorized by the Committee on Bioethics at ENES, Unit Leon, National Autonomous University of Mexico. The tissue was stored in PBS and 2% antibiotic. The sample was washed twice with PBS and 2% chlorhexidine. The primary cell culture was performed using explants of 1x1mm approximately. The tissue was suspended in α-MEM medium supplemented with 20% FBS, heat inactivated, 100IU/ml penicillin G and 100mg/ml streptomycin sulfate. Cells were incubated at 37°C with an atmosphere of 5% CO₂ and 95% humidity for two weeks changing the culture medium every third day until exponential growth was observed. HGFs have an in vitro life expectancy of approximately 40 PDL (population doubling level).

The cells were detached enzymatically from the culture dish with 0.25% trypsin-EDTA 0.025% -2Na for each experiment. After the primary cell culture was established, the experiments were carried out using DMEM+10% FBS and antibiotics.

Adhesion assay and cell proliferation.
Cells were inoculated at a density of 2x10⁶ cells/ml in each of the Ti and Zr samples and incubated at room temperature (23°C) for 60 minutes. Samples were washed twice with PBS to remove nonadherent cells. In the case of cell proliferation, cells were incubated for 24 hours more at 37°C with 5% CO₂. The number of viable cells attached and proliferated on the surfaces was determined by MTT method. Subsequently, 0.2mg/ml of MTT reagent was mixed in DMEM +10% FBS and incubated for 3 hours at 37°C with 5% CO₂. Formazan was completely dissolved with DMSO, transported to a 96-well plate and analyzed at 540nm in a microplate reader (Thermo Fisher Scientific, St. Louis, Missouri, USA). In cell adhesion and proliferation Ti plates were used as control value. Assays were performed in triplicate from three independent experiments.

Statistic analysis
The mean, standard deviation and percentage were calculated. All data were tested with Kolmogorov-Smirnov tests of normality (Lilliefors), Kruskal-Wallis test, and multiple comparisons using Mann-Whitney (SPSS, Chicago, IL, USA). Statistical significance was considered at p<0.05 and a 95% confidence interval.

Figure 1. Micrographs of atomic force microscopy.
A) 2-D Zr, B) 3-D Zr, C) 2-D Ti and D) 3-D Ti.
RESULTS.

Topography of the Zr plates showed a higher roughness (Ra=0.39 μm) (Fig. 1A and 1B) than Ti (Ra=0.049 μm) (Fig. 1C and 1D). Ti samples showed an almost flat surface with some sipes and micropores, in contrast, Zr plates showed a roughened surface with a high presence of micropores.

Quantification of HGF adhesion was significantly higher (p<0.05) in the Ti with 42% (±18.2%) more when compared to adherent cells in Zr samples (Fig. 2A), while the proliferation revealed a greater number of cells on the surfaces of Ti with 37% (±27.4%) showing no statistical differences (Fig. 2B), having a similar biological response between both surfaces.

DISCUSSION.

The aim of modifying the surfaces of the implants is not only to adapt them to the demands to avoid the negative effects of implanted materials into the surrounding tissue, but to improve the interaction between the material and tissues. The use of current technologies that modify the surface of the implants has become a trend in marketing and production of new implants, creating different morphologies and chemical treatments to improve and accelerate osseointegration.

For a long time, osseointegration was identified as a local factor that could interfere with the success of dental implants. Now, it is known that not only the osseointegration of dental implants contributes to the integration of the adjacent tissues, but also of the soft tissues adjacent to the implant.

Long-term stability of dental implants, the biological seal of soft tissues and implant interface are important features for the clinical success of oral rehabilitation. The transmucosal part of the dental implant requires sufficient attachment of connective tissue and inhibition of bacterial invasion. Conventionally, surfaces in contact with soft tissues, particularly the abutment, are designed with smooth surfaces to prevent bacteria from adhering easily.

The aim of this research was to quantify in vitro the HGF response on Ti and Zr surfaces by cell adhesion and proliferation. The results shown here indicate that initially Ti surfaces significantly increase HGF adhesion by 42%, while cell proliferation, comparing both surfaces showed...
no difference in the number of proliferated cells, suggesting that the biological response in both surfaces after 24 hours can yield similar and comparable results. The type of material with which dental implants are manufactured is critical to their success, also the topography of the implant surface influences integration\(^\text{14}\) qualitatively and quantitatively\(^\text{14}\).

However, the review of the current literature showed partly contradictory results. Yamano et al.\(^\text{17}\) identified the topography of the surfaces as an important modulator of fibroblast behavior, both in vitro and in vivo, demonstrating that smooth Zr surfaces promote more adhesion and proliferation of fibroblasts. The difference between their results and this research could be caused by the polishing of the surfaces. Furthermore, Pae et al.\(^\text{16}\) suggest that microporosity allows greater diffusion and alignment of fibroblasts, when compared to the smooth surface. The controversy lies not only in the topography of the Zr surfaces. Velasco-Ortega et al.\(^\text{8}\) found that osteoblastic cells cultured on rougher Ti surfaces differ faster than on smoother surfaces, however, Xiaohui Rausch-fan et al.\(^\text{11}\) suggested that the smoothest surfaces are the ideal surfaces for better adhesion and cell proliferation. Furthermore, Esfahanizadeh et al.\(^\text{18}\) showed that the difference between the adhesion on Zr and Ti surfaces is not significant, which is line with the findings of this study.

The method reported here is an easy and reproducible technique for the study of the interaction between Ti and Zr cells and surfaces. The method of rapid colorimetry by MTT is based on the identification of the metabolic activity of the cells adhered and proliferated on the surface. The results shown in this study are more representative than those previously published by direct counting with scanning electron microscopy\(^\text{19}\) or enzymatic detachment with trypsin and ultrasonic vibration\(^\text{20}\). Such methods may underestimate the number of cells adhered and proliferated to surfaces. However, this study is preliminary and it is necessary to investigate the kinetics of the interaction, the specific morphological and functional relationship of the cells on surfaces, as well as controlling the expression of different genes associated with cell adhesion and proliferation, such as integrin expression on surfaces.

**CONCLUSION.**

Although initially Ti showed increased cell adhesion on the surface after 24h, Zr samples showed a similar proliferation. Therefore, both surfaces have comparable biological response.
REFERENCES.

1. Vishwakarma A, Sharpe P, Shi S, Ramalingam M. Stem Cell Biology and Tissue Engineering in Dental Sciences. 1st Ed. London: Elsevier Ltd; 2015.
2. Anderson JM. Biological responses to materials. Annu Rev Mater Res. 2001;31:81–110.
3. Gómez-Florit M, Ramis JM, Xing R, Taxt-Lamolle S, Haugen HJ, Lyngstadhaas SP, Monjo M. Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces. J Periodontal Res. 2014;49(4):425–36.
4. Chug A, Shukla S, Mahesh L, Jadwani S. Osseointegration—Molecular events at the bone–implant interface. J Oral Maxillofac Surg Med Pathol. 2013;25(1):1–4.
5. Babu RSA, Ogle O. Tissue response: biomaterials, dental implants, and compromised osseous tissue. Dent Clin North Am. 2015;59(2):305–15.
6. Lee TJ, Ueno T, Nomura N, Wakaba K, Park J. Engineering biocompatible implants: A Rapidly Evolving Practice. New York: InTech; 2011. pp. 83–108.
7. Fage SW, Muris J, Jakobsen SS, Thysen JP. Titanium: a review on exposure, release, penetration, allergy, epidemiology, and clinical activity. Contact Dermatitis. 2016;74(6):323–45.
8. Velasco-Ortega E, Alfonso-Rodríguez CA, Monsalve-Guil L, España-López A, Jiménez-Guerra A, Garzón I, Alaminos M, Gil FJ. Mater Sci Eng C Mater Biol Appl. 2016;64:1–10.
9. Möller B, Terheyden H, Açıl Y, Purcz NM, Hertrampf K, Tabakov A, Behrens E, Wiltfang J. A comparison of biocompatibility and osseointegration of ceramic and titanium implants: an in vivo and in vitro study. Int J Oral Maxillofac Surg. 2012;41(5):638–45.
10. Altuna P, Lucas-Taulé E, Gargallo-Albiol J, Figueras-Álvarez O, Hernández-Alfaro F, Nart J. Clinical evidence on titanium-zirconium dental implants: a systematic review and meta-analysis. Int J Oral Maxillofac Surg. 2016;45(7):842–50.
11. An N, Rausch-fan X, Wieland M, Matejka M, Andrukho O, Schedle A. Initial attachment, subsequent cell proliferation/viability and gene expression of epithelial cells related to attachment and wound healing in response to different titanium surfaces. Dent Mater. 2012;28(12):1207–14.
12. Contreras RG, Adachi K, Yokote Y, Sakagami H, Hibino Y, Nakajima H, Shimada J. Quantification of enhanced osteoblastic adhesion to ultraviolet-treated titanium plate. In Vivo. 2010;24(4):519–23.
13. Bauer S, Schmuki S, Von Der Mark K, Park J. Engineering biocompatible implant surfaces: Part I: Materials and surfaces. Prog Mater Sci. 2013;58(3):261–326.
14. Anil S, Anand PS, Alghamdi H, Jensen JA. Dental implant surface enhancement and osseointegration. Implant Dentistry: A Rapidly Evolving Practice. New York: InTech; 2011. pp. 83–108.
15. Kwon D, Choi HJ, Lee H, Lee JW, Weber HP, Pae A. Cellular viability and genetic expression of human gingival fibroblasts to zirconia with enamel matrix derivative (Emdogain®). J Adv Prosthodont. 2014;6(5):406–14.
16. Pae A, Lee H, Kim HS, Kwon YD, Woo YH. Attachment and growth behaviour of human gingival fibroblasts on titanium and zirconia ceramic surfaces. Bio Med Mater. 2009;4(2):025005.
17. Yamano S, Ma AK, Shanti RM, Kim SW, Wada K, Sukotjo C. The influence of different implant materials on human gingival fibroblast morphology, proliferation, and gene expression. Int J Oral Maxillofac Implants. 2011;26(6):1247–55.
18. Esfahanizadeh N, Motalebi S, Daneshparvar N, Akhoundi N, Bonakdar S. Morphology, proliferation, and gene expression of gingival fibroblasts on Laser-Lok, titanium, and zirconia surfaces. Lasers Med Sci. 2016;31(5):863–73.
19. Ueno T, Yamada M, Suzuki T, Minamikawa H, Sato N, Hori N, Takeuchi K, Hattori M, Ogawa T. Enhancement of bone-titanium integration profile with UV-photofunctionalized titanium in a gap healing model. Biomaterials. 2010;31(7):1546–57.
20. Iwasa F, Hori N, Ueno T, Minamikawa H, Yamada M, Ogawa T. Enhancement of osteoblast adhesion to UV-photofunctionalized titanium via an electrostatic mechanism. Biomaterials. 2010;31(10):2717–27.