



## ORIGINAL ARTICLE

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## 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= 0.39 $\mu$ m) than Ti (Ra= 0.049 $\mu$ m). 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.

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### 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 dentition<sup>1</sup>. Implant placement is a complex, multidisciplinary task involving various disciplines and requiring a deep knowledge of periodontics, surgery and prosthetics<sup>2</sup>.

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 structure<sup>1</sup>; soft tissue integration to provide a biological seal between the oral cavity and the implant<sup>2</sup> and the appropriate biocompatibility of the tissue at systemic and local level<sup>3</sup>. 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 surface<sup>3</sup>, which also determines the type of coating and rehabilitation procedures<sup>4</sup>.

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 response<sup>5</sup>.

Biomaterials commonly used for dental implants are manufactured from metal (titanium and its variants) and ceramic materials covered by porcelain (aluminum and zirconium oxide)<sup>6</sup>. Ti is widely used because it has proven to be a biocompatible and bio-inert material, stable and very well tolerated by soft tissues<sup>7</sup>. 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 osseointegration<sup>8</sup>.

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 implant<sup>9</sup>. However, due to the lack of information on their performance in the short and long term<sup>10</sup>, 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 surface<sup>11</sup>, 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 ( $\alpha$ -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 1 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 1 $\mu$ m 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 $\mu\text{m}$  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  $\alpha$ -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% CO<sub>2</sub> 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<sup>12</sup>.

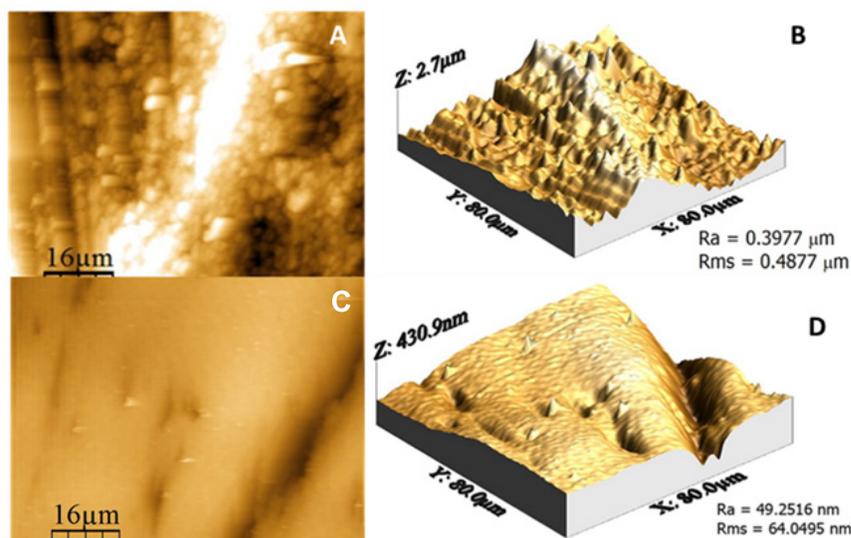
### Adhesion assay and cell proliferation.

Cells were inoculated at a density of 2x10<sup>6</sup> cells/ml in each of the Ti and Zr samples and incubated at room temperature (23°C) for 60 minutes<sup>12</sup>. 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% CO<sub>2</sub>. 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% CO<sub>2</sub>. 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.

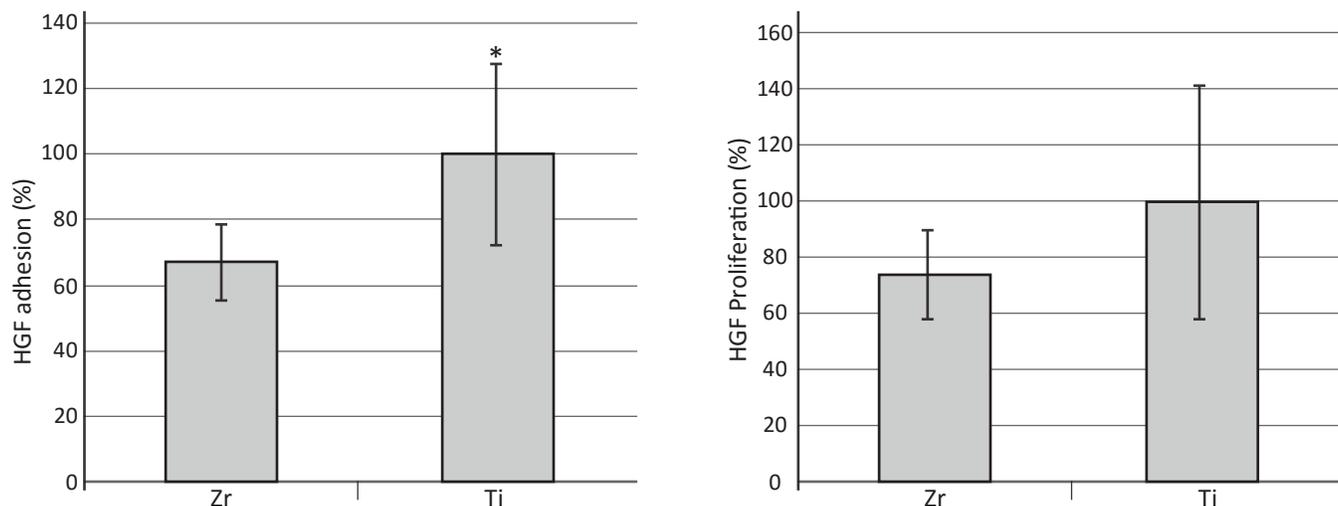
### Statistic analysis

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.

**Figure 2.** HGF adhesion and proliferation on Ti and Zr.

A) HGF Adhesion B) HGF Proliferation. Fibroblasts were inoculated at  $2 \times 10^6$  cells/ml. Viable cells adhered and proliferated and were determined by MTT bioassay and analyzed at 540nm by microplate spectrophotometer. The values of Ti surfaces were used as value control. Bars represent the mean  $\pm$  standard deviation in triplicate of three independent experiments. \*  $p < 0.05$  multiple Mann-Whitney comparisons.

## RESULTS.

Topography of the Zr plates showed a higher roughness ( $R_a = 0.39 \mu\text{m}$ ) (Fig. 1A and 1B) than Ti ( $R_a = 0.049 \mu\text{m}$ ) (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% ( $\pm 18.2\%$ ) 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% ( $\pm 27.4\%$ ) 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<sup>13</sup>. 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<sup>14</sup>.

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<sup>11</sup>.

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<sup>15</sup>. Conventionally, surfaces in contact with soft tissues, particularly the abutment, are designed with smooth surfaces to prevent bacteria from adhering easily<sup>16</sup>.

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<sup>14</sup> qualitatively and quantitatively<sup>14</sup>.

However, the review of the current literature showed partly contradictory results. Yamano *et al.*<sup>17</sup> 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.*<sup>16</sup> 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.*<sup>8</sup> found that osteoblastic cells cultured on rougher Ti surfaces differ faster than on smoother surfaces, however, Xiaohui Rausch-fan *et al.*<sup>11</sup> suggested that the smoothest surfaces are the ideal surfaces for better adhesion and cell proliferation. Furthermore, Esfahanizadeh *et al.*<sup>18</sup> 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<sup>19</sup> or enzymatic detachment with trypsin and ultrasonic vibration<sup>20</sup>. 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.

## Respuesta de fibroblastos a la adhesión inicial y proliferación en superficies de titanio y zirconio.

**Resumen:** Introducción: En las últimas décadas, los implantes dentales se han posicionado como una de las mejores opciones de restauración dental integral; su colocación es una tarea multidisciplinaria que requiere una sólida comprensión de los principios biológicos, periodontales, quirúrgicos y protésicos. Objetivo: El objetivo de este estudio fue cuantificar *in vitro* la adhesión y proliferación de la respuesta de fibroblastos gingivales humanos (HGF) en superficies de titanio (Ti) en contraste con superficies de zirconia (Zr). Metodología: Se observaron las muestras de Ti y Zr bajo microscopía de fuerza atómica (AFM). Los HGF fueron inoculados en cada muestra para determinar la adhesión y proliferación celular. El reactivo MTT se mezcló con medio DMEM y se inoculó en cada placa, el formazán se disolvió con dimetilsulfóxido y se analizó a 540nm en un espectrofotóme-

tro de microplaca. El ensayo se realizó con tres experimentos independientes. Los datos fueron analizados con pruebas de Kolmogorov-Smirnov (Lilliefors), pruebas de Kruskal-Wallis y comparaciones de Mann-Whitney. Resultados: La topografía de las placas de Zr mostraron una mayor rugosidad ( $Ra=0.39\mu m$ ) en comparación con las de Ti ( $Ra=0.049\mu m$ ). La cuantificación de la adhesión de HGF fue significativamente mayor ( $p<0.05$ ) en el Ti, mientras que la proliferación no mostró diferencias estadísticamente significativas entre los grupos. Conclusión: Es importante mencionar que, a pesar de que el Ti mostró inicialmente una mayor adhesión celular sobre la superficie, después de 24 hrs las muestras de Zr mostraron una proliferación similar; lo que demuestra que ambas superficies poseen una respuesta biológica comparable.

**Palabras clave:** *Implantes dentales, Titanio, Zirconia, Biomateriales, Fibroblastos gingivales humanos.*

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