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# Effects of $TiO_2$ NPs Coating-Titanium for Fibroblast Adhesion

## Efectos del Recubrimiento de NPs de TiO2 Sobre Placas de Titanio para la Adhesión De Fibroblastos

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**ABSTRACT:** The objective of this study was to determine the effects of coating nanoparticles of titanium dioxide ($TiO_2$ NPs) and irradiation -UV on plates of titanium (Ti) for the adhesion and proliferation of human gingival fibroblasts (HGF). A total of 15 Ti plates were divided into three groups (n = 5); (i) control Ti, (ii) experimental: Ti+$TiO_2$ NPs, (iii) experimental: Ti+$TiO_2$ NPs+UV. The plates were analyzed with atomic force microscopy (AFM) and the roughness (Ra and Rmax) was determined. UV irradiation was performed for 20 min. HGF were subcultured in DMEM+10 % fetal bovine serum (FBS) at 37 °C with 5 % $CO_2$. 2x106 cells/mL were inoculated on the plates and incubated for 1 h and washed with phosphate buffer saline (PBS). In the case of cell proliferation, cells were incubated for further 24 h more. Cell viability was determined with the MTT method, the formazan was dissolved with dimethylsulfoxide (DMSO) and analyzed at 540 nm. Experiments were performed of three independent experiments and data were analyzed by Kruskall-Wallis and multiple comparison of Mann-Whitney test. The surface topography of samples corresponded as follow: Ti (Ra= 0,492 μm y Rms= 0.640 μm), Ti+NPs $TiO_2$, (Ra= 0.55 μm y Rms= 0.714 μm), respectively. The coating with $TiO_2$ NPs significantly ($p < 0.05$) increased the adhesion and proliferation of HGF compared with the group. The modification of Ti plates by coated with $TiO_2$ NPs significantly increased adhesion and proliferation of HGF with the formation of a hydrophilic surface which favors the humectancy. This treatment may be reported here convenient to accelerate osseointegration of dental implants based titanium.

**KEY WORDS: titanium, $TiO_2$ nanoparticles, UV irradiation, cell adhesion, human gingival fibroblast.**

## INTRODUCTION

Much research and considerable effort has been devoted to changing the topography and the chemical composition of the surfaces of titanium implants to improve their osteoconductive and osseointegration. The coating titanium implants with a bioactive material to improve their osseointegration is the subject of multiple investigations, however it has not been resolved completely (Li *et al*., 2015).

The sterilization of dental implants plays an important role in the osseointegration of same (Albresktsson *et al*., 1986). The radiofrequency discharge luminescent (Hartman *et al*., 1989) and irradiation of ultraviolet light (UV) (Riley *et al*., 2005) ensure a neat and hydrophilic surface of titanium implants thus contributing to their bone integration (Garcia-Contreras, 2011).

On the other hand, limited function osseointegration of Ti is related to the presence of galvanic phenomena as reported for many studies (Vanegas Acosta *et al*., 2010; Aparicio *et al*., 1998; Cao *et al*., 2011). It has been proposed that the modification of the surface, considering the variation of the roughness and the formation of an anodic layer to prevention release or formation products, can generate a cytotoxic environment that leads to cell death and subsequent tissue presenting a lack of integration

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(Taborelli *et al*., 1997; Mendonça *et al*., 2008). Some research has determined that there is an increase in surface properties of the Ti plate to be coated with titanium dioxide ($TiO_2$) nanoparticles (NPs) which allow the Ti to be more stable when was in contact with oral tissues and decrease the release of metal ions causing tissue necrosis at periimplant zones which leads in turn to failure in treatment (Vargas-Reus *et al*., 2012; Park *et al*., 2009; Wold, 1993).

Photocatalysis induced by UV irradiation also has been used to treat the surface of Ti plates achieving favorable results to significantly increase cell proliferation based on the number of viable cells and the consumption of amino acids from the culture medium, specifically, arginine and glutamine (Young, *et al*., 2005), MTT method has been reported as a reproducible assay for determinate the viable cell number on the attached cells on the Ti plates (Contreras *et al*., 2010).

Therefore this paper aims to investigate the effects of changing the topography of the surface coating $TiO_2$ NPs and chemical composition of Ti plates using UV irradiation to enhance cell adhesion and proliferation of human gingival fibroblasts (HGF) by MTT colorimetry method.

## MATERIAL AND METHOD

**Preparation of Ti plates**. Plates type 1 commercially pure (99.5 %) (Tokuriki, Melters, Tokyo, Japan) of 20x20x0.05 mm were obtained. The samples were embedded in epoxy resin and automatically polished (160-200 rpm; Buehler, Lake Bluff, IL, USA) with water sandpaper of different grain sizes, #400, 800, 1000, 1500 and 200 (Fuji Star, Sankyo, Rikagaku, Okegawa, Japan) and finally diamond suspension of 0.05-1 μm with felt (Chemomet, Buehler, Lake Bluff, IL, USA). Then, the plates were removed from the epoxy resin and ultrasonically cleaned with distilled water, 99.5 % ethanol and 99.5 % acetone for 10 min and blow dried at room temperature. Ti plates were be divided into three experimental groups (i) Control (Ti), (ii) Experimental (Ti+$TiO_2$ NPs), (iii) Experimental (Ti+$TiO_2$ NPs+UV) (n= 5 gp). All samples were packaged and sterilized by autoclave. The application of UV irradiation on the titanium plates was done with a UV lamp (Germicidal T8, General Electric, Lynn, Massachusetts, USA), 254 nm wavelength and 55 mW, for 20 min. Plates were reused throughout the experiments after being polished and sterilized.

**Ti plates coated with $TiO_2$ NPs**. The Ti plates were coated with a layer of $TiO_2$ NPs (Nanopowder, titanium IV oxide, anatase, Sigma Aldrich, St. Louis, Missouri, USA) at a concentration of 32 mm previously vortexed (vortex-Genie 2 Daigger MIXERS laboratory, Vernon Hills, Illinois USA) and sonicated (BRANSON 2510 ultrasonic Danbury, Fairfield, USA). It is important to mention that Ti plates were exposed to UV irradiation lamp for 20 min previous to coated the surface with $TiO_2$ NPs. NPs were placed onto the Ti plates and spin coating (HOLMARC, HO-TH-05, Kochi, India) in 5 cycles (15 s 250 rpm, 15 s 500 rpm, 15 s 1000 rpm, 15 s 1500 rpm, 15 s 2000 rpm). For experimental group (Ti+$TiO_2$ NPs+UV) a further UV irradiation was carried out for 20 min previously to inoculate the cells onto the surface.

**Ti plates surface topography**. The surface was evaluated with atomic force microscopy (Nanosurf FlexAFM, Liestal, Switzerland) to consider average roughness (Ra) and maximum roughness height (Rmax) within a Sample Length area of 80x80 μm using the tapping mode according to ISO 4287:1997: Geometrical Products Specifications (GPS)-Surface texture: Profile method.

**Cell culture**. Human gingival fibroblasts (HGF-1, ATCC, CRL-2014) were subcultured in modified eagle medium of Dulbecco (DMEM, Life Technologies, Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Life Technologies, Gibco, Carlsbad, CA, USA) heat inactivated, 100 IU/mL penicillin G and 100 μg/ml streptomycin sulfate (Life Technologies, Gibco) in 10-cm polyethylene dishes under the laminar flow (Lumistell LH-120, Biotechnical Bajio, Celaya, Mexico) and incubated at 37 °C in an atmosphere of 5 % of $CO_2$. Cells were washed with saline phosphate buffer (PBS) (pH 7.4) and resuspended enzymatically dish polyethylene trypsin 0.25 % EDTA-2Na with (Life Technologies, Gibco) for each experiment.

**Cell adhesion and proliferation**. HGF cells were cultivated onto the plates at $2x10^6$ cells/mL. The cell attachment was left for 1 h at room temperature (24 °C). Subsequently, the culture medium was removed from each plate and washed twice with PBS to remove the unattached cells. In case of cell proliferation, cells were incubated for another 24 h at 37 °C with 5 % de $CO_2$. MTT reagent (Sigma Aldrich, St. Louis, Missouri, USA) were mixed in DMEM (0.2 mg/mL) and incubated

for 4 h at 37 °C in 5 % of $CO_2$, the formazan was dissolved with dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, Missouri, USA). The dissolved formazan was transported to 96-wells culture dish. The plates were analyzed at 540 nm in a microplate spectrophotometer (Thermo scientific St. Louis, Missouri, USA). The assay was conducted in three independent experiments (n= 15 per group).

**Statistical analysis**. For descriptive statistics; average, standard deviation and percentage were calculated. All data were analyzed with normality tests Kolmogorov-Smirnov (Lilliefors), Kruskal-Wallis and Mann-Whitney multiple comparisons. Statistical significance was considered at 0.05 with a confidence interval of 95 %.

## RESULTS

The surface topography of samples correspond for Ti plates with Ra= 0.492 μm and Rms= 0.640 μm (Fig. 1A and B), and Ti+$TiO_2$ NPs, Ra= 0.55 μm and Rms= 0.714 μm (Fig. 1C and D), respectively. The group coated with $TiO_2$ NPs with or without UV treatment significantly ($p < 0.05$) increased adhesion and proliferation of HGF compared to the control group The plates coated with $TiO_2$ NPs and UV irradiation significantly promotes the formation of a hydrophilic surface which favors humectancy and therefore adhesion and cell proliferation ($p < 0.05$) compared with unmodified titanium plates (Fig. 2A and B).

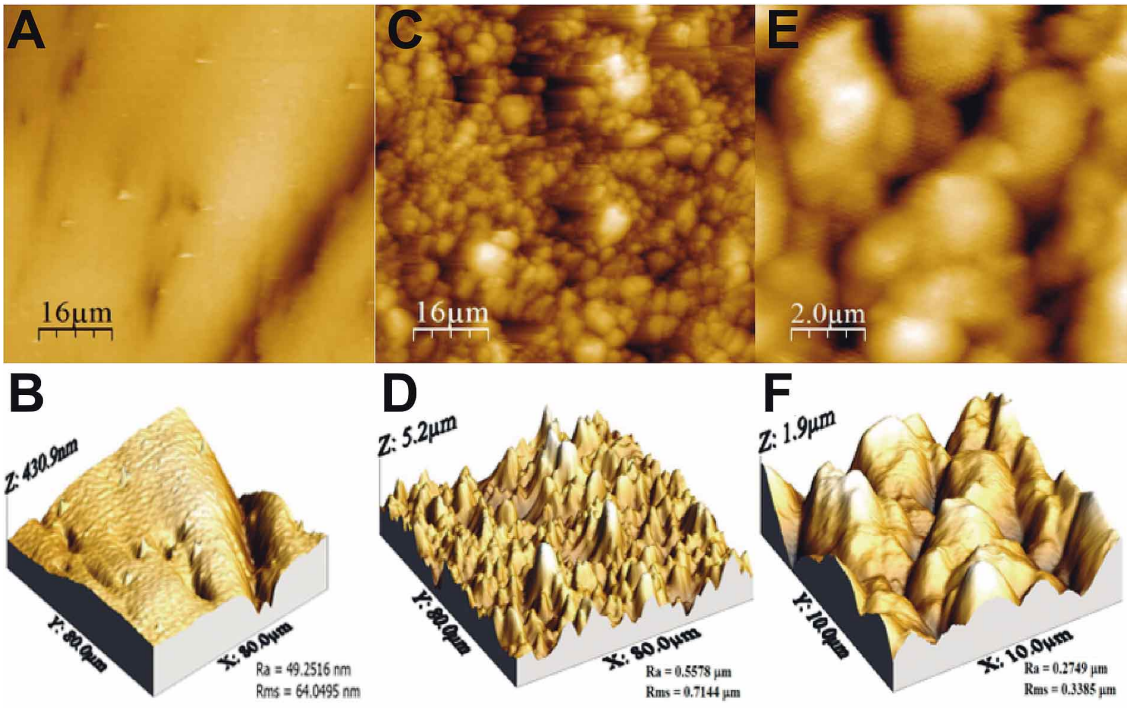


Fig. 1. Topography of titanium plates with and without $TiO_2$ NPs. A) Ti 2D, B) Ti 3D, C) Ti+NPs $TiO_2$ 2D, D) Ti+NPs $TiO_2$ 3D, E) Ti+NPs $TiO_2$ 2D, F)Ti+NPs $TiO_2$ 2D.

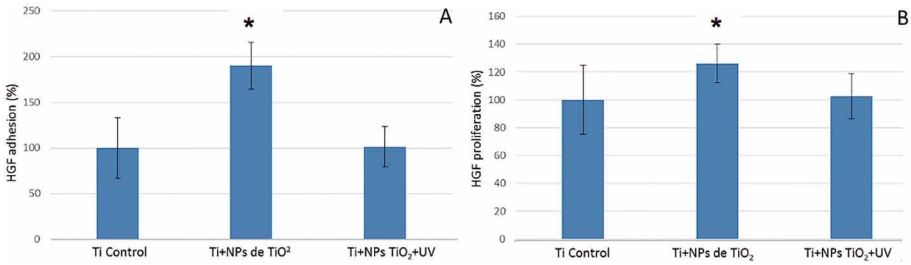


Fig. 2. Adhesion and proliferation of HGF on the Ti and coated with $TiO_2$ NPs. A) Adhesion at room temperature for one hour. B) Proliferation for 24 h of incubation. Data represents mean ± standard deviation of three independent experiments (n= 15 per group). *p <0.05 based on multiple comparisons of Mann-Whitney test.

## DISCUSSION

In the present study the results found that there was a significant increase in adhesion and proliferation of HGF on titanium surfaces treated with $TiO_2$ NPs with or without UV irradiation, compared with the control group. The $TiO_2$ NPs and UV light produces greater humectancy of plates, immediately, making the titanium surface a highly hydrophilic area which favors the uniform dispersion of the cells over the entire surface, then after 24 h the number of viable cells is comparable to plates with or without UV, suggesting that NPs increase the adhesion and proliferation and UV is beneficial for immediate and integration humectancy in all titanium implants as similarly as previously reported (Contreras *et al*.).

Photocatalytic properties of $TiO_2$ NPs increase cell adhesion compared to pure titanium which was fully demonstrated in our experiment, however, the use of $TiO_2$ NPs was more successful both adhesion and proliferation of fibroblasts. The first report of the use of photocatalysis back to 1970 (Fujushima & Honda, 1972) several studies have focused on the antibacterial effects and sterilization with UV irradiation (Xu *et al*., 2006; Koseki *et al*., 2009). The present study confirms further that the UV light irradiation on the titanium plates for 20 min improves adhesion and proliferation of fibroblasts and significantly enhances the cell attachment when $TiO_2$ NPs were present on the Ti plates.

Conversely, the wavelength and emitted watts of UV light are important for increasing cell adhesion, also the distance between the plates of Ti and UV irradiation source and dose of photocatalysis.

Since the method of rapid colorimetric MTT is based on metabolic cell activity, the present study results can be more reliable than those reported by Sawase *et al*. (2008), where counting the number of adherent cells was performed by laser microscopy and specialized software; on the other hand Onuki *et al*. (2010) detached with trypsin adherent cells and cell counting was performed with hemocytometer and finally Koseki *et al*. (2009) isolated cells from the surface with trypsin and ultrasonic waves, those methods previously reported are ineffective for correct estimate of the number of viable cells. However, the MTT method has a limitation, minimum cells still remain on the surface, underestimation due to lack of contact time MTT reagent with cells, therefore, in future research an increase in the incubation time of the reagent with cells is recommended. To solve this problem researchers have reported the amino acid consumption reported by Onuki *et al*., or evaluate the perimeter attachment (Contreras *et al*.), both are appropriate choice for monitoring cellular activity.

Future research should be focused to include more details on the kinetics between cells and surfaces of Ti, which involves time dose-response UV irradiation, monitor gene expression of fibroblast cells, differentiation mature fibroblastic cells posteriorly to the surface modification, the use of animal models, in addition to assessing the hydrophilicity by the contact angle of the Ti plates and the surface roughness correlate with adhesion and cell proliferation.

The main constraint during the realization of the experiments was the lack of stability of the media and solutions on the surface of the titanium plates due to changes in its surface which could have diminished some values. As suggestions for future experiments I propose to make adjustments to the titanium surface to maintain this stability and successful results with minimal variability.

## CONCLUSION

Based on the results obtained it showed that the surface modification of titanium plates using the $TiO_2$ coating NPs significantly increased adhesion of fibroblasts and proliferation thereof to 24 h. This treatment may be an advisable to accelerate the process of osseointegration of titanium dental implant option.

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**ORNELAS-PONCE, A.; AMEZCUA-ROMERO, J.; ACOSTA-TORRES, L. S. & GARCIA-CONTRERAS, R.** Efectos del recubrimiento de NPs de $TiO_2$ sobre placas de titanio para la adhesión de fibroblastos. *Int. J. Odontostomat., 10(2)*:237-242, 2016.

**RESUMEN:** El objetivo fue determinar los efectos del recubrimiento con nanopartículas de dióxido de titanio ($TiO_2$ NPs) e irradiación UV sobre placas de titanio (Ti) para la adhesión y proliferación de fibroblastos gingivales humanos (FGH). Un total de 15 placas de Ti se dividieron en tres gru-

pos (n= 5); (i) control Ti, (ii) experimental Ti+NPs $TiO_2$, (iii) experimental: Ti+NPs $TiO_2$+UV. Las placas fueron analizadas en microscopía de fuerza atómica (MFA) y se determinó la rugosidad (Ra y Rmax). La irradiación con UV se realizó durante 20 min. FGH fueron subcultivados en DMEM+10 % de suero fetal bovino a 37 °C con 5 % de $CO_2$. 2x106 células/mL fueron inoculadas sobre las placas e incubadas durante 1 h, se lavaron con solución salina de buffer fosfato. En el caso de la proliferación celular, las células se incubaron por 24 h más. La viabilidad celular se determinó con el método de MTT, el formazan fue disuelto con dimetilsulfoxido y se analizó a 540 nm. Los experimentos se realizaron a partir de tres experimentos independientes y los datos se analizaron por Kruskall-Wallis y por comparación múltiple de Mann-Whitney. La topografía de la superficie de las muestras correspondio de la siguiente manera: Ti (Ra= 0,492 μm y Rms= 0,640 μm), Ti+NPs $TiO_2$, (Ra= 0,55 μm y Rms= 0,714 μm), respectivamente. El recubrimiento con NPs $TiO_2$ aumentó significativamente la adhesión y proliferación de HGF en comparación con el grupo de Ti control ($p < 0,05$). La modificación de la superficie de las placas de Ti recubiertas con NPs $TiO_2$ aumentó significativamente la adhesión y proliferación de HGF con la formación de una superficie hidrófila que favorece la humectancia. Este tratamiento aquí informado tal vez sea un método conveniente para acelerar el proceso de la osteointegración de los implantes dentales a base de titanio.

**PALABRAS CLAVE: titanio, nanopartículas de $TiO_2$, irradiación UV, adhesión celular, fibroblastos gingivales humanos.**

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