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Effects of alkaline treatment for fibroblastic adhesion on titanium

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ABSTRACT

Background: The surface energy of titanium (Ti) implants is very important when determining hydrophilicity or hydrophobicity, which is vital in osseointegration. The purpose of this study was to determine how Ti plates with an alkaline treatment (NaOH) affect the adhesion and proliferation of human periodontal ligament fibroblasts (HPLF).

Materials and Methods: *In vitro* experimental study was carried out. Type I commercially pure Ti plates were analyzed with atomic force microscopy to evaluate surface roughness. The plates were treated ultrasonically with NaOH at 5 M (pH 13.7) for 45 s. HPLF previously established from periodontal tissue was inoculated on the treated Ti plates. The adhered and proliferated viable cell numbers were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method for 60 min and 24 h, respectively. The data were analyzed using Kruskal–Wallis tests and multiple comparisons of the Mann–Whitney U-test, P value was fixed at 0.05.

Results: The mean roughness values equaled 0.04 μm with an almost flat surface and some grooves. The alkaline treatment of Ti plates caused significantly ($P < 0.05$) more pronounced HPLF adhesion and proliferation compared to untreated Ti plates.

Conclusion: The treatment of Ti plates with NaOH enhances cell adhesion and the proliferation of HPLF cells. Clinically, the alkaline treatment of Ti-based implants could be an option to improve and accelerate osseointegration.

Key Words: Cells, periodontal ligament, sodium hydroxide, titanium, wettability

INTRODUCTION

The clinical success of titanium (Ti) implants in odontology is directly related to the osseointegration process, which results from the interaction between tissues and implant surfaces.[1] Integration occurs when the implant comes into contact with the oral fluids and tissues after implantation in a host bond. This bilateral interaction will depend on the intensity of the mechanical and chemical irritation and the nature of the implant surface.[2] The increasing biocompatibility of Ti surfaces has been continuously researched.[3] The characteristics of the implant surface are one of the factors that affect the rate of osseointegration. Some studies have determined that surface modifications include physical and morphological aspects such as roughness, porosity, topography,[4] photocatalysis,[5] and surface functionalized with extracellular matrix

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peptide[6] to enhance the cellular adhesion. Moreover, chemical treatment alterations involving plasma, saline solutions, anodic oxidation, and hydroxyapatite[7] have improved cellular aggregation.[8]

The early phase of osseointegration occurs during the 1st week after implant positioning as this stage has the highest failure probability.[3] The reactions induce biofilm formation, which regulates cellular responses. During the 1st week, bone formation starts as a result of osteoblasts differentiating, as well the production of osteogenic factors, cytokines, and growth factors. Primary bone includes trabeculae of woven bone, which will be replaced by lamellar bone and marrow bone that will in turn be reabsorbed for new bone formation.[9] The initial phase of osseointegration leads to the aggregation of osteogenic cells to the implant surface, which should allow cellular proliferation and differentiation until maturation.[9]

The surface energy of the implant plays an important role in determining the hydrophilicity or hydrophobicity of the surface, which is important in osseointegration. In general, when a positive charge exists, the surface acquires hydrophilic characteristics, which offers advantages in bone integration. Initially, some essential plasma proteins are absorbed by these surfaces, helping to enhance cell grouping and the expression of osteogenic genes.[5,9]

Studies concerning the hydrophilicity of dental implants have become increasingly important as bone response increases osteogenic potential and osseous tissue formation in early bone response and therefore osteointegración.[10-12] Current studies in human bone marrow-derived mesenchymal cells have demonstrated an increase in the gene expression levels of the markers and transcription runt-related factor 2, with bone sialoprotein also observed.[11] *In vitro* tests show higher cell adhesion on hydrophilic surfaces such as photocatalysis[5,13] compared to anodized coating with calcium phosphate on hydrophobic surfaces.[11] As mentioned above, an increase in the hydrophilicity of the surface represents an increase in the adhesion of bone and gingival fibroblast cells not only on the apical surface of the implant but also on the accession of the peri-implant soft tissue. Both mechanisms would enhance osseointegration and reduce torsional forces.[14,15] The processes used to maintain surface hydrophilicity may include acid etching,[16] sandblasting and in saline storage, as well as treatment with sodium hydroxide.[15] The purpose of this study was to determine the effects of alkaline treated (sodium hydroxide) Ti plates in the adhesion of human periodontal ligament fibroblasts (HPLF) and proliferation of the cells using a rapid colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay to determine cell viability of the cell attachment.

MATERIALS AND METHODS

Sample preparation

An *in vitro* experimental study was performed at the Escuela Nacional de Estudios Superiores (ENES) Unidad León, Universidad Nacional Autónoma de México (UNAM), Interdisciplinary Research Laboratory, Nanostructures and Biomaterials area. Type I commercially pure Ti (Ti: 99.5%; Tokuriki, Chiyoda-Ku, Tokyo, Japan) was used to prepare 10 × 10 × 0.5 mm (n = 5 per group, control and experimental samples) plates because it is the most used in the manufacture of dental implants for biological properties, which were placed in epoxy resin and polished with an automatic polisher (160–200 rpm; Buehler, Lake Bluff, USA) and #400, 800, 1000, 1500, and 2000 water sandpaper. The surface was finished with a polycrystalline diamond suspension of 0.05–1 μm using a polishing cloth (Chemomet, Buehler, Lake Bluff, IL, USA). After polishing, the samples were removed and washed with distilled water and ethanol for 5 min in ultrasound and then blow dried. Samples were packed and sterilized using an autoclave treatment.[13]

Surface topography

The surface was evaluated with atomic force microscopy (AFM) (Nanosurf FlexAFM, Liestal, Switzerland) to consider average roughness (R_a) and maximum roughness height within a sample length (R_{max}) area of 80 × 80 μm using the tapping mode according to ISO 4287:1997: Geometrical products specifications-surface texture: Profile method.

Alkaline surface treatment

Alkaline surface treatment was implemented based on published protocol. Experimental samples were intended for alkaline treatment modification with 0.5 M of NaOH (pH 13.7) (23°C). The samples were soaked in NaOH solution and sonicated for 45 s. The samples were then dried at room temperature (23°C) in the chamber fluid for 5 min, and the cells were inoculated on the control and experimental Ti plates.[3]

Cell culture

HPLFs were obtained through periodontal tissue extraction of the third molar from an 18-year-old patient, with the prior written informed consent from the parents. The project was authorized by the Bioethics Committee of ENES, ENES Unidad León, UNAM, Unidad León. The extracted tooth was suspended in phosphate buffered saline (PBS); the tissue was washed twice with PBS and suspended in Dulbecco's Modified Eagle's Medium (DMEM Life Technologies, Gibco, Carlsbad, CA, USA) supplemented with 20% of heat inactivated Fetal Bovine Serum (FBS, Life Technologies, Gibco), 100 µ/ml penicillin G, and 100 mg/ml of sulfate streptomycin (Life Technologies, Gibco). The cells were incubated at 37°C with an atmosphere of 5% CO_2 for 2 weeks for exponential growth with changes in the growth medium every 3rd day. HPLF have an *in vitro* life expectancy of about 40 population doubling level. Cells were detached using 0.25% trypsin and 0.025% ethylenediaminetetraacetic acid-2Na in PBS for each experiment.[13]

Assay of cell adhesion and proliferation

HPLF cells were subcultured as adherent cells in DMEM supplemented with 10% FBS and antibiotics. Cells were inoculated in each experimental and control Ti plate at 2 × 10^6 cells/ml. Cell inoculation was performed immediately after the alkaline treatment; 150 µl was placed in each plate and allowed to incubate at room temperature (23°C) for 60 min. Plates were washed twice with 150 µl of PBS to remove unattached cells. In case of cell proliferation, cells were incubated for a further 24 h at 37°C with 5% CO_2. The viable adherent and proliferated cells were determined by MTT method. Briefly, 0.2 mg/ml of a MTT reagent was dissolved in DMEM + 10% FBS for 3 h. The formazan was dissolved with 100 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich Co, St. Louis, MO, USA); cells were collected from Ti plates and were transported to a 96-well plate, and then analyzed at 540 nm using a microplate reader (Thermo Fisher Scientific). Data were reproduced in triplicate from three independent experiments.[13]

Statistical analysis

The mean, standard deviation, and percentage were calculated. All data were subject to test for normality data by Kolmogorov–Smirnov (Lilliefors), Kruskal–Wallis tests, and multiple Mann–Whitney U-test comparisons. The significance was considered at $P < 0.05$ with a 95% interval confidence.

RESULTS

Surface roughness

The values obtained for R_a and R_{max} of the Ti plates were 0.04 ± 0.02 µm and 1.83 ± 0.10 µm, respectively. Figure 1a and b exhibits two-dimensional and three-dimensional AFM micrographs of polished Ti plates with an almost flat surface and some scratches and grooves on the 80 × 80 µm analyzed area.

Cell attachment and proliferation

The treatment of Ti plates with sodium hydroxide significantly enhanced ($P < 0.05$) the adhesion of HPLF. The alkaline treatment caused more pronounced cell attachment ($P < 0.05$) for the HPLF line compared to the Ti control plates [Figure 2a] with 30% more adhered cells. The quantification of proliferated cells after incubation for 24 h showed 90% higher attached cells ($P < 0.01$) in Ti plates treated with NaOH, indicating an active proliferation, compared to control plates [Figure 2b].

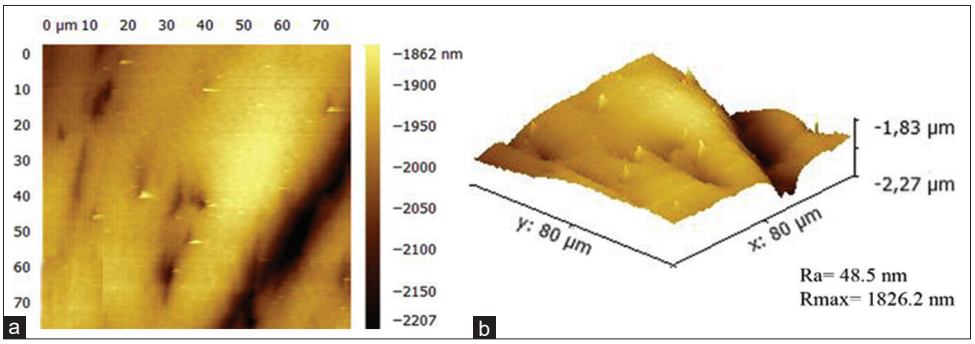

Figure 1: Micrographs of atomic force microscopy roughness of titanium plate surface. The roughness values corresponded to Ra and R_{max} of a 80 x 80 µm area using the tapping mode in accordance with ISO 4287:1997. (a) Two-dimensional, (b) Three-dimensional micrograph.

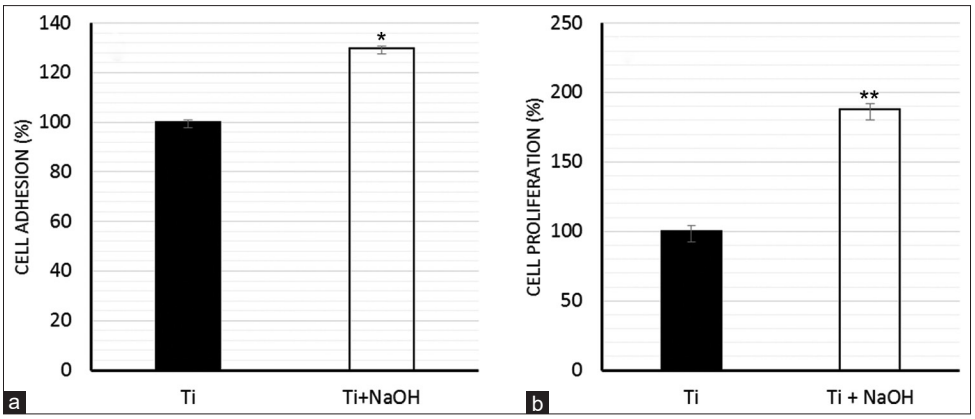

Figure 2: Effects of alkaline treatment on human periodontal ligament fibroblasts cell adhesion (a) and proliferation (b). Cells were subcultured as adherent cells in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum. They were then inoculated on titanium plates at 2×10^6 cells/ml for 60 min for adhesion (a) and a further 24 h of incubation at 37°C with 5% CO_2 for proliferation. The viable adherent and proliferate cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (0.2 mg/ml) and analyzed at 540 nm using a microplate. Reader values represent a mean ± standard deviation. Data were reproduced in triplicate from three independent experiments. *$P < 0.05$, **$P < 0.01$ based on the Mann–Whitney U-test.

DISCUSSION

The treatment with NaOH has shown that this modification supports fast and homogeneous protein absorption through hydrophilicity and the electrostatic attraction to cells.[17] Hydrophilic surfaces in Ti implants and cell response have been studied for their osteolytic potential *in vitro*[11] and *in vivo*[1] conditions. This treatment proposes increasing cell activation, protein intake, and the forming of fibrin matrix during the early phases of osseointegration for clinical success. This study focused on the effects of alkaline treatment with NaOH as a hydrophilic surface conditioner for cell attachment. Consistent with our findings, cell adhesion is usually greater on hydrophilic surfaces treated with different materials and methods including alkaline treatment.

On the other hand, similar to our investigation, alkaline treatment was effective for mouse osteoblast cell exposed to a 5 M solution of NaOH with 20% higher attachment and proliferation[18] of the MC3T3-E1 mouse preosteoblast cell line to the hydrophilic surface.[19] Similarly, another assay has reported better effects in the use of sodium hydroxide 0.05 M in monocyte and osteoblastic cell adhesion and platelet activation.[17] Chemical modification with hydrochloric/sulfuric acids increased osteogenic differentiation in five primary human osteoblast cell lines.[20] Proliferation results in our research coincide with other assays without significant difference after 24 h[3,21] of incubation.

Surface roughness plays a very important role in cell attachment and bone proliferation. Varying surface roughness alters adhesion results. This may be attributed to the additional use of surface modifications such as microgrooves as studies have reported that hydrophilicity on the surface may have a stronger effect on bone formation.[1]

The main findings of the research support evidence that sodium hydroxide treatment allows hydrophilicity on the surface of Ti plates and enhances the attachment of cells and proliferation of HPLF by increasing the number of viable cells, coinciding with a previous study.[13] The protocol reported here seems to be a reproducible and rapid method for determining the attached cell number using a metabolically active *in vitro* assay as previously reported.[13] It is based on the conversion of yellow, water-soluble MTT to a purple, water-insoluble end product formazan using mitochondrial dehydrogenase. The amount of formazan formed is proportional to the number of metabolically active cells.[22] The key steps in the assay are to incubate cells with MTT, extract the formazan crystals from the cells, dissolve the formazan, and measure absorbance between 540 and 570 nm.[23]

This study is preliminary, and one of its limitations was the recovery of a number of total attached cells on Ti plates with DMSO, suggesting that a few cells can remain on the surface. Future experiments will require a longer incubation time (4 or 5 h) of the MTT reagent to enhance the formazan and recover the total number of cells with DMSO. Future research should be focused on evaluating the duration of the sprouting

process and the morphological features that arise, such as counting the round and flat cells on the metal surface and measuring the attached area and perimeter of cells in the plates as previously reported.[5] Moreover, an *in vivo* evaluation is necessary to obtain more scientific evidence. The treatment of Ti plates with NaOH enhanced the cell attachment and proliferation of HPLF culture cells over the surface. These data help to illustrate the possible effect of hydrophilicity in the process of bone formation on a Ti surface.

CONCLUSION

The treatment of Ti plates with NaOH enhances significanlty the cell adhesion and the proliferation of HPLF cells. Clinically, the alkaline treatment of Ti-based implants could be an option to improve and accelerate osseointegration.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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