

Response of osteoblastic cells to titanium submitted to three different surface treatments

Resposta de células osteoblásticas ao titânio submetido a três diferentes tratamentos de superfície

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ABSTRACT: In the complex process of bone formation at the implant-tissue interface, surface properties are relevant factors modulating osteoblastic function. In this study, commercially pure titanium (cp Ti) samples were prepared with different surface characteristics using chemical attack with a sulfuric acid/hydrochloric acid based solution (treatment A); chemical attack plus anodic oxidation using phosphoric acid (treatment B); and chemical attack plus thermal oxidation followed by immersion in a sodium fluoride solution (treatment C). The samples were characterized by scanning electron microscopy (SEM), contact profilometry and contact angle. The biological performance of the prepared surfaces was evaluated using mice osteoblastic cell cultures for up to 21 days. Cells seeded on the different titanium samples showed similar behavior during cell attachment and spreading. However, cellular proliferation and differentiation were higher for samples submitted to treatments A and C ($p \leq 0.05$; $n = 3$), which were less rough and showed surface free energy with smaller polar components.

DESCRIPTORS: Titanium; Surface properties; Osteoblasts; Biocompatible materials.

RESUMO: No complexo processo de formação óssea na interface implante-osso, as propriedades de superfície são um importante fator modulador da função osteoblástica. No presente estudo, foram preparadas amostras de titânio comercialmente puro (cp Ti) com diferentes propriedades de superfície por meio de ataque químico com solução à base de ácido sulfúrico/clorídrico (tratamento A); ataque químico seguido de oxidação anódica com ácido fosfórico (tratamento B); e ataque químico seguido de oxidação térmica e imersão em fluoreto de sódio (tratamento C). As chapas foram caracterizadas por meio de microscopia eletrônica de varredura (MEV), perfilometria e ângulo de contato. O comportamento de células osteoblásticas de camundongo foi acompanhado por três semanas. As células cultivadas sobre os diferentes substratos de titânio apresentaram um modelo de comportamento similar durante as etapas de adesão e espalhamento. No entanto, a proliferação e a diferenciação celulares foram maiores nas amostras submetidas aos tratamentos A e C, que se apresentaram menos rugosas e com energia livre de superfície com menores componentes polares.

DESCRIPTORES: Titânio; Propriedades de superfície; Osteoblastos; Materiais biocompatíveis.

INTRODUCTION

Titanium is a material widely used for implants because of its biocompatibility⁵. This material has high corrosion resistance, suitable mechanical properties and can be easily produced in many different shapes and textures. A key question in most applications of titanium is how the material influences, and is influenced by the biological response that results from the contact between biomaterial and biological systems.

Titanium is a reactive metal that forms, spontaneously, in the air, water or any other electrolyte, a thin native oxide film, which is responsible for titanium biocompatibility. Surface properties of an implant play a critical role in this process as bone cells can recognize and respond to surfaces⁶. Surface modification aims to accelerate osseointegration and includes hydroxyapatite (HA) coatings, mechanical blasting (using either Al_2O_3 or

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TiO₂ particles), anodic oxidation and acid-etching processes (different concentrations of H₂SO₄, HF, HNO₃ and HCl). Recently, some authors have reported on significantly improved bone tissue reactions by modification of surface oxide properties of titanium implants^{1,19} or ion release². Chemical modification of titanium implant surface is of particular interest because it may enhance osseointegration without embedding surface contaminants, such as grit particles²¹.

The biological response of osteoblastic cells includes cell attachment, cell growth and functional activity⁷. Concerning osteoblastic differentiation and metabolism, the results reported in the literature are somewhat controversial. While some papers show that increasing surface roughness enhances *in vitro* osteoblastic differentiation and inhibits cell proliferation^{3,11,12}, others^{1,6,13,15,18,19} indicate that proliferation can be improved on specimens submitted to higher surface roughness. These results suggest that there are other aspects that also modulate proliferation, differentiation and extracellular matrix production of osteoblastic cells *in vitro*.

In this study, the behavior of mice osteoblastic cells seeded on titanium submitted to three different surface treatments was evaluated in terms of cell attachment, cell proliferation (MTT assay) and cell differentiation (alkaline phosphatase – ALP activity). Additionally, a correlation between cell response and surface properties was established.

MATERIALS AND METHODS

Sample preparation

A commercially pure titanium (ASTM grade 2) (Titanews, São Paulo, Brazil) sheet with 1 mm in thickness was cut in 10 mm x 10 mm squares (for cell culture) and 20 mm x 10 mm rectangles (for sample characterization). According to the surface treatment employed, three groups of samples were obtained, as shown in Table 1. The aim of treatment A was to create surface roughness, while samples B and C had an additional treatment to increase oxide thickness. Anodization and thermal oxidation samples were based on the findings of Sena *et al.*¹⁸ (2003) and Vanzillotta²⁰ (2003), respectively. C samples were additionally immersed in a NaF solution to allow fluoride incorporation, which seems to be beneficial to bone formation^{8,9}. After surface treatments, the samples were cleaned using acetone (Merck, São Paulo, Brazil) and distilled water (Quimis Q-341, Diadema, Brazil) in an ultrasonic cleaner and then sterilized by autoclaving.

TABLE 1 - Surface treatments employed.

Sample	Treatment
A	18% HCl + 48% H ₂ SO ₄ (55°C – 60 s)
B	First step: 18% HCl + 48% H ₂ SO ₄ (55°C – 60 s); Second step: anodic oxidation (IPRJ, Nova Friburgo, Brazil) with 8% H ₃ PO ₄ in ethanol solution (20 V – 10 min)
C	First step: 18% HCl + 48% H ₂ SO ₄ (55°C – 60 s); Second step: thermal oxidation (Quimis, Diadema, Brazil) (450°C – 60 min); Third step: immersion in 4% NaF (40 min)

HCl, H₂SO₄, H₃PO₄, NaF (Merck, São Paulo, Brazil).

Scanning electron microscopy (Zeiss DSM 940A, Oberkochen, Germany), operating at 15 kV was employed for qualitative evaluation of the titanium topography. Surface roughness was measured with a contact stylus profilometer (Perthometer, Perthen, Gottingen, Germany) over a 250 μm² area, using a Gaussian filter (80 μm) to exclude form and waviness characteristics from the roughness measurements, according to the DIN 4768 standard. For each group, three specimens were employed, and at least 10 measurements were made on different regions of each sample. Three parameters were used: Ra (the average surface roughness, or average deviation, of all points from a plane fit to the test part of the surface); Rq (the square-root of the average of the measured height deviations) and Rz (the average absolute value of the five highest peaks and the five lowest valleys over the evaluation length).

Contact angle measurements were used to calculate the surface free energy (SFE) of the titanium samples. Contact angles (θ) on the sterilized titanium surfaces were measured with a goniometer (Ramé-Hart Instrument Co., Netcong, NJ, USA) by the captive air bubble method. SFE components were obtained by the equation¹⁴:

$$(1 + \cos \theta) \gamma_l = 2[(\gamma_s^d \gamma_l^d)^{1/2} + (\gamma_s^p \gamma_l^p)^{1/2}]$$

where: θ is the contact angle between the liquid and the captive air bubble;

subscript s and l are the solid and liquid surfaces, respectively;

γ^d stands for the dispersion component of the total surface energy (γ);

γ^p is the polar component.

Water (γ_l = 72.8 mJ/m²; γ_l^d = 21.9 mJ/m²; γ_l^p = 51.0 mJ/m²) and glycerol (γ_l = 64.0 mJ/m²;

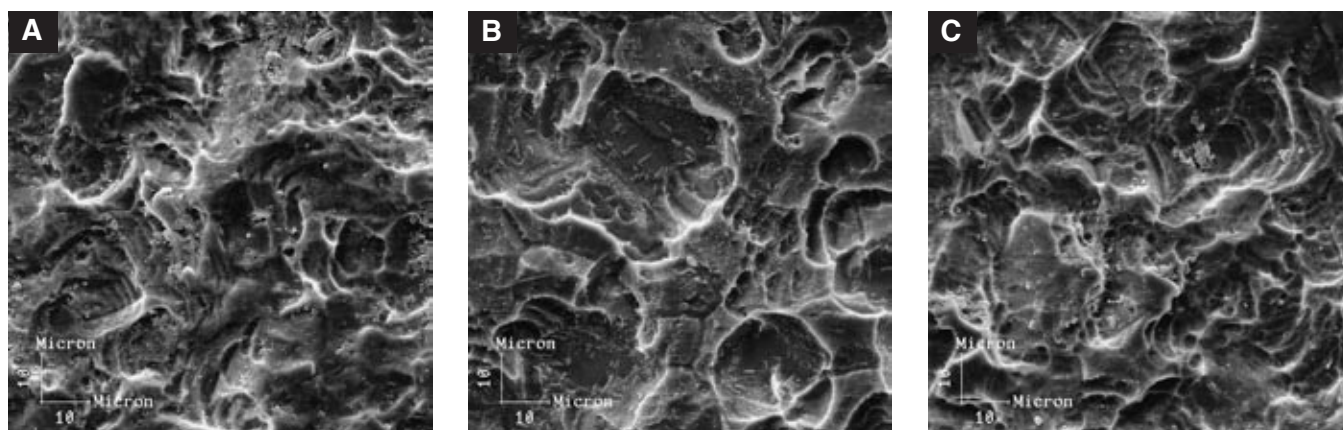


FIGURE 1 - SEM micrographs (1,000 X) of the titanium samples submitted to: **A)** Treatment A; **B)** Treatment B; **C)** Treatment C.

$\gamma_1^d = 34.0 \text{ mJ/m}^2$; $\gamma_1^p = 30.0 \text{ mJ/m}^2$) were used as the standard liquids.

Cell culture

Mice were obtained from our breeding colony. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Committee for the Use of Experimental Animals of our Institution.

Mice osteoblastic cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) containing 10% of fetal bovine serum. Primary cultures were maintained until near confluence and, at the 6th passage, adherent cells were enzymatically released (0.04% trypsin – Sigma, St. Louis, MD, USA – and 0.025% collagenase – Sigma, St. Louis, MD, USA) and seeded in 24-well plates (Sigma, St. Louis, MD, USA) at a density of 10^4 cells/cm². Incubation was carried out in a humidified atmosphere of 95% air and 5% CO₂ at 37°C, and culture medium was changed twice a week. Cells were cultured on both control (glass – Elzividros, Rio de Janeiro, Brazil) and titanium samples for up to 21 days.

Cell attachment was observed by fluorescence microscopy 5 and 24 hours after incubation. Titanium substrates (n = 3 for each treatment) were stained with 4-6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA), and the number of adherent cells was determined for each of the three specimens.

As for cell viability/proliferation evaluation, the cells were cultured for 7, 14 and 21 days on titanium specimens and analyzed using the MTT (Sigma, St. Louis, MD, USA) assay. This method is

based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by viable cells to a purple formazan salt. The cells were incubated with 50 μl of MTT for 4 hours at 37°C. The dark blue formazan crystals were dissolved with acid sodium dodecyl sulfate (0.1 N HCl SDS, Merck, Darmstadt, Germany) and then they were kept in the acid solution overnight to ensure complete dissolution of crystals. The absorbance was determined at 595 nm in an ELISA reader (Bio-Rad, Hercules, USA).

Alkaline phosphatase (ALP) activity was assayed by the hydrolysis of *p*-nitrophenyl phosphate (Sigma, St. Louis, MD, USA) in alkaline buffer solution (pH 10.3) (Sigma, St. Louis, MD, USA), and colorimetric determination of the product (*p*-nitrophenol) was carried out at 405 nm (ELISA reader, Bio-Rad, Hercules, USA). ALP activity was calculated from a standard curve and results were expressed in nanomoles of *p*-nitrophenol produced per minute.

Statistical analysis

For cell culture analysis, data are presented from one of two replicate experiments, both of which yielded comparable results. For any given experiment, each data point represents the mean \pm standard deviation of three replicates. Statistical analysis was done by one-way analysis of variance (ANOVA), and statistical differences between the three samples were determined by Tukey's multiple comparison *post hoc* test. Only *p* values ≤ 0.05 were considered significant.

RESULTS

SEM micrographs (Figure 1) show a similar rough topography for the three samples (A, B, C)

TABLE 2 - Roughness parameters for titanium samples.

Parameter (μm)	A (acid)	B (acid + anodic oxidation)	C (acid + thermal oxidation + NaF)
Ra	2.78	3.04	2.21
Rq	3.51	10.73	2.70
Rz	28.60	156.34	17.74

Ra (the average surface roughness, or average deviation, of all points from a plane fit to the test part of the surface); Rq (the square-root of the average of the measured height deviations); Rz (the average absolute value of the five highest peaks and the five lowest valleys over the evaluation length).

TABLE 3 - Polar (γ_s^p) and dispersive (γ_s^d) components of the surface free energy (SFE) calculated by Owens-Wendt's method.

Sample	γ (mJ/m ²)	γ_s^p (mJ/m ²)	γ_s^d (mJ/m ²)
A	56	8	48
B	43	14	29
C	42	9	33

studied. Table 2 shows the roughness parameters (Ra, Rq and Rz) for the titanium samples and Table 3 shows surface free energy (γ_s) and their polar (γ_s^p) and dispersive (γ_s^d) components calculated by solving simultaneous equations (Owens-Wendt approach¹⁴).

Ra was not significantly affected by the treatments used for surface preparation. However, the two other roughness parameters (Rq and Rz) were significantly different when samples A and C were compared to sample B ($p < 0.05$), with A and C showing smaller roughness values. Surfaces submitted to the treatments of samples A and C showed quite similar and smaller polar components.

Data on cell attachment are presented in Graph 1, which shows that attachment was not affected by surface topography either after 5 h or after 24 h. However, cell attachment was higher on control (glass) substrates ($p \leq 0.05$; $n = 3$) than on Ti samples. The results of cell viability/proliferation and of ALP activity are presented in Graph 2 and Graph 3, respectively. Results concerning cell viability/proliferation evaluated by MTT assay showed that cell growth was not affected by surface treatment after 7 days in culture, where osteoblastic cells proliferated very well on all substrates. The behavior in control cultures showed cell growth during two weeks, decreasing after that ($p \leq 0.001$; $n = 3$). Treatment B resulted in a high decrease in the cell growth rate after 14 days and maintenance of this rate after 3 weeks ($p \leq 0.05$; $n = 3$).

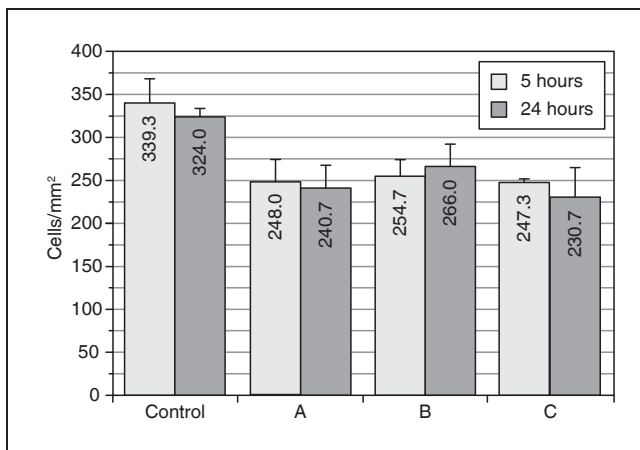
Alkaline phosphatase (ALP), which is produced during osteoblastic differentiation, diffuses into circulation and is used as a serum biochemical marker of bone formation. Until day 14, ALP activity was reduced in all samples, however, samples A and C exhibited a greater activity, which remained at higher levels at day 21 when compared to control and sample B surfaces ($p \leq 0.05$; $n = 3$) indicating an osteoblastic differentiation stimulus.

DISCUSSION

The present work showed that different chemical treatments may produce similar topographies of cp Ti surfaces (Figure 1). This result was quite expected as oxide films are very thin (from 30 to 80 nm)²⁰ and may not influence topography on a micrometer scale.

Surfaces submitted to treatments A and C exhibited quite similar and smaller polar components. Hallab *et al.*¹⁰ (2001) demonstrated that SFE is a more relevant surface characteristic than surface roughness for cellular adhesion strength and proliferation, and that surface energy components of the various tested materials proved to be related to cellular adhesion strength: poor correlation was observed between the dispersive component of SFE and adhesion strength when compared to the polar component of SFE. Similar correlations were observed by Ponsonnet *et al.*¹⁶ (2003).

The biological performance of the titanium samples was evaluated in terms of their ability to allow cell attachment, proliferation and differentiation. Cell adhesion is one of the initial events essential to subsequent proliferation and differentiation of bone cells before bone tissue formation. Consequently, many *in vitro* evaluations of cell adhesion on substrates with various roughness levels have been performed in order to identify the main surface properties influencing cell response to implant surface⁵. Cell adhesion is a very specific parameter and describes the relative adherence of



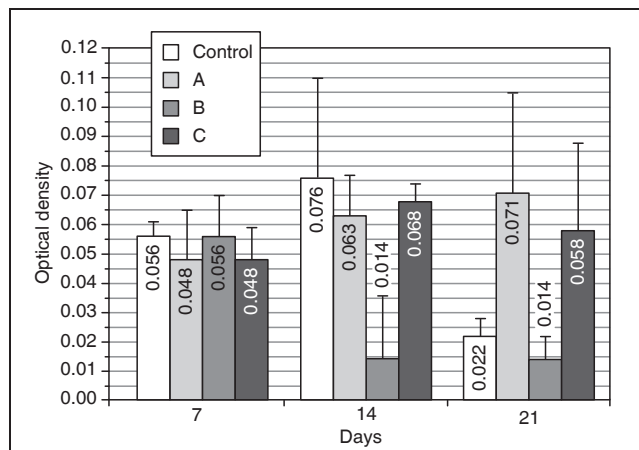
GRAPH 1 - Number of adherent osteoblast cells after 5 and 24 hours in culture on treated Ti and control surfaces. All data are reported as mean \pm standard deviation ($n = 3$).

a cell to its substrate, generally at an early stage of culture when cells are directly in contact with the material surface⁴.

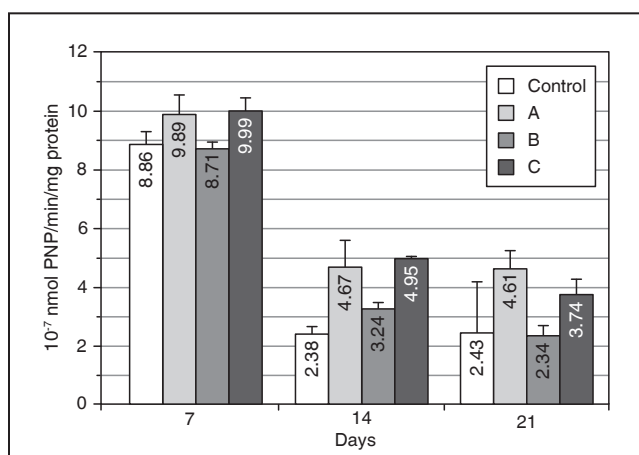
The similarity observed in all samples after 5 or 24-hour inoculation can have two explanations: 1) it is possible that, after the initial hours, osteoblastic cells can easily attach, spread over the entire surface, and then start to die because of growth limitation (in this case, after 24 hours, cell number could be reduced to the same value observed after 5 hours); or 2) osteoblastic cell attachment could be the same after both studied times. An *in vitro* evaluation of cell adhesion 24 hours after seeding is not sufficient to anticipate the future integration of a material several weeks after implantation.

Analyzing data of the ALP assay, the most relevant aspect was that, after 14 days, osteoblastic differentiation showed to be affected by surface treatment, and the best behaviors were observed in samples A and C. These observations support the results concerning cell viability/proliferation evaluated by the MTT assay, and indicate a better behavior of osteoblasts seeded on substrates A and C.

Findings of the present work show that proliferation is unfavorably affected by increasing surface roughness, in agreement with other studies^{3,12,17} that observed better cell responses on more organized surfaces. This behavior was probably due to the slightly smoother surface and to the surface free energy with smaller polar components.



GRAPH 2 - Viability/proliferation (MTT assay) of mice osteoblastic cells on control and treated surfaces after 7, 14 and 21 days. All data are reported as mean \pm standard deviation ($n = 3$).



GRAPH 3 - Differentiation (alkaline phosphatase activity) of mice osteoblastic cells on control and treated surfaces after 7, 14 and 21 days. All data are reported as mean \pm standard deviation ($n = 3$). PNP: *p*-nitrophenol.

CONCLUSIONS

Cell differentiation/viability/proliferation was higher for Ti samples submitted to treatments A (chemical attack with a sulfuric acid/hydrochloric acid based solution) and C (chemical attack plus thermal oxidation followed by immersion in sodium fluoride solution). Therefore, these treated surfaces seem to provide a better environment for mice osteoblastic cell integration. These results suggest that the treatments used in the present study may support favorable biological responses *in vivo*.

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