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# Biological characterization of implant surfaces - in vitro study

*Caracterização biológica de superfície de titânio - estudo in vitro*

Priscilla Barbosa Ferreira SOARES[a*], Camilla Christian Gomes MOURA[a], Huberth Alexandre da ROCHA JÚNIOR[a], Paula DECHICHI[a], Darceny ZANETTA-BARBOSA[a]

[a]Faculdade de Odontologia, UFU – Universidade Federal de Uberlândia, Uberlândia, MG, Brazil

## Resumo

**Objetivo:** Avaliar o desempenho biológico de ligas de titânio grau IV submetidos a diferentes tratamentos de superfície – jateamento e duplo ataque ácido (Superfície experimental 1; Exp1, NEODENT) e superfície com aumento na molhabilidade (Superfície experimental 2; Exp2, NEODENT) em resposta preliminar de diferenciação e maturação celular. **Material e método:** Foram plaqueados osteoblastos imortalizados sobre discos de titânio de Exp1 e Exp2 e como controle o poço da placa de cultura sem disco (C). Empregou-se ensaios de viabilidade celular (MTT) em 4 e 24 horas (n = 5), adesão celular em 4 horas (n = 5), dosagem de proteínas totais e fosfatase alcalina normalizada em 4, 7 e 14 dias (n = 5). Os dados foram analisados por ANOVA em fator único seguido de teste de Tukey. **Resultado:** Os valores de viabilidade celular foram: 4h: C– 0,32±0,01$^{A}$; Exp1 – 0,34±0,08$^{A}$; Exp2– 0,29±0,03$^{A}$. 24h: C– 0,43±0,02$^{A}$; Exp1– 0,39±0,01$^{A}$; Exp2– 0,37±0,03$^{A}$. A contagem de adesão celular foi: C– 85±10$^{A}$; Exp1– 35±5$^{B}$; Exp2– 20±2$^{B}$. Os valores de proteínas totais foram: 4d: C– 40±2$^{B}$; Exp1– 120±10$^{A}$; Exp2– 130±20$^{A}$. 7d: C– 38±2$^{B}$; Exp1– 75±4$^{A}$; Exp2– 70±6$^{A}$. 14 d: C– 100±3$^{A}$; Exp1– 130±5$^{A}$; Exp2– 137±9$^{A}$. Os valores de fosfatase alcalina normalizada foram: 4d: C– 2,0±0,1$^{C}$; Exp1– 5,1±0,8$^{B}$; Exp2– 9,8±2,0$^{A}$, 7d: C– 1,0±0,01$^{C}$; Exp1– 5,3±0,5$^{A}$; Exp2– 3,0±0,3$^{B}$, 14 d: C– 4,1±0,3$^{A}$; Exp1– 4,4±0,8$^{A}$; Exp2– 2,2±0,2$^{B}$. Letras diferentes representam diferenças estatísticas. **Conclusão:** As superfícies testadas apresentaram comportamento diferenciado na dosagem de fosfatase alcalina normalizada traduzindo que Exp2 está relacionado com processo de indução de diferenciação celular e Exp1 relacionado com processo de mineralização.

**Descritores:** Superfície de titânio; ensaio colorimétrico mitocondrial; citotoxidade celular.

## Abstract

**Objective:** Evaluate the biological performance of titanium alloys grade IV under different surface treatments: sandblasting and double etching (Experimental surface 1; Exp1, NEODENT); surface with wettability increase (Experimental surface 2; Exp2, NEODENT) on response of preliminary differentiation and cell maturation. **Material and method:** Immortalized osteoblast cells were plated on Exp1 and Exp2 titanium discs. The polystyrene plate surface without disc was used as control group (C). Cell viability was assessed by measuring mitochondrial activity (MTT) at 4 and 24 h (n = 5), cell attachment was performed using trypan blue exclusion within 4 hours (n = 5), serum total protein and alkaline phosphatase normalization was performed at 4, 7 and 14 days (n = 5). Data were analyzed using one-way ANOVA and Tukey test. **Result:** The values of cell viability were: 4h: C– 0.32±0.01$^{A}$; Exp1– 0.34±0.08$^{A}$; Exp2– 0.29±0.03$^{A}$. 24h: C– 0.43±0.02$^{A}$; Exp1– 0.39±0.01$^{A}$; Exp2– 0.37±0.03$^{A}$. The cell adhesion counting was: C– 85±10$^{A}$; Exp1- 35±5$^{B}$; Exp2– 20±2$^{B}$. The amounts of serum total protein were 4d: C– 40±2$^{B}$; Exp1– 120±10$^{A}$; Exp2– 130±20$^{A}$. 7d: C– 38±2$^{B}$; Exp1– 75±4$^{A}$; Exp2– 70±6$^{A}$. 14 d: C– 100±3$^{A}$; Exp1– 130±5$^{A}$; Exp2– 137±9$^{A}$. The values of alkaline phosphatase normalization were: 4d: C– 2.0±0.1$^{C}$; Exp1– 5.1±0.8$^{B}$; Exp2– 9.8±2.0$^{A}$. 7d: C– 1.0±0.01$^{C}$; Exp1– 5.3±0.5$^{A}$; Exp2– 3.0±0.3$^{B}$. 14 d: C– 4.1±0.3$^{A}$; Exp1– 4.4±0.8$^{A}$; Exp2– 2.2±0.2$^{B}$. Different letters related to statistical differences. **Conclusion:** The surfaces tested exhibit different behavior at dosage of alkaline phosphatase normalization showing that the Exp2 is more associated with induction of cell differentiation process and that Exp1 is more related to the mineralization process.

**Descriptors:** Titanium surface; mitochondrial colorimetric assay; cell cytotoxicity.

## INTRODUCTION

Reestablishment of esthetic and chewing functions of the stomatognathic system using osseointegrated dental implants has become the new paradigm in oral and maxillofacial rehabilitation of edentulous individuals[1]. Tissue and cellular responses to the composition of the implant surface, specifically to the physical and chemical characteristics, have determined the clinical success

of the procedures performed[2-4]. The osseointegration of titanium may be influenced by various mechanisms, especially the surface properties represented by the chemical characteristics such as structural composition; mechanical, such a residual stress; and, physical, such as wettability. Thus, it is known that interactions with the molecules and cells of the liquids with which the implants come into contact generate changes in the surface properties[5].

The development of implant surfaces with textures that mimic the microenvironment in which the cells live seeks to promote events related to contact osteogenesis, in addition to greater mechanical stability[6]. Modifications related to the surface topography of titanium implants, among them the blasting of abrasive particles such as oxides of Al2O3, SiO2 and TiO2; surface etching with HF and HNO3, among other acids; association and techniques such as blasting followed by etching; anodization; ion beam deposition; and, use of bioactive components such as growth factors, proteins or calcium and phosphorous ions have been widely studied[5,7-10]. Although there is still no consensus regarding treatment capable of producing the ideal implant surface, capable of accelerating the process of new bone formation[11] in the literature, in vitro research indicates that moderately rough surfaces with around 1.5µm Sa and 50% Sdr result in better performance and bone healing[7]. The surface topography of the implants influences the wettability of these and other parameters related to the osteogenesis process such as viability, growth curve, cellular adhesion and expression of proteins from the bone matrix[3,12,13]. In vivo studies have also confirmed the advantages of rough surfaces, such as greater bone-implant contact, acceleration of neo-osteogenesis and greater torque for their removal, when compared to machined implants[9,14-16].

However, prior to proposing commercial distribution of titanium implants with chemical and physical modifications, it is necessary to evaluate the effects, both in vitro and in vivo, of the incorporation of these substances on the osteogenesis process. Therefore, the purpose of this study was to evaluate the biological performance of grade IV titanium alloys submitted to different surface treatments in initial response to cellular differentiation and maturation, on the parameters of adhesion, viability and alkaline phosphatase production.

## MATERIAL AND METHOD

### *Characterization of the Surfaces*

Discs of commercially pure, grade IV titanium with dimensions of 6.0mm diameter X 2.0 mm thickness were produced in the Neodent® Materials Laboratory (Curitiba, PR, Brazil). After machining, the discs were submitted to blasting and a double acid etching process. Half of these discs were submitted to physico-chemical processing which adds hydrophilicity to the surface, resulting in greater wettability thereof. Thus, 2 experimental groups were created: Exp1 (titanium discs submitted to blasting and double acid etching) and Exp2 (titanium discs with increased wettability).

### *Osteoblast Culture on the Surfaces*

The cells of the SAOS-2 osteoblast lineage (Banco de Células Rio de Janeiro, Rio de Janeiro, RJ, Brasil) grew in culture bottles containing osteogenic medium until they acquired confluence. They were enzymatically released, transferred to tubes containing Eagle medium modified by 5% Dulbecco (DMEM) (Sigma Chemical Co., St. Louis, MO, USA), centrifuged for 5 minutes at 1000x*g*, counted by hemocytometer and plated on titanium discs (Exp1 and Exp2). The bottom of the polystyrene plate (Coastar Corp., Cambridge, MA, USA) was used as the control (Group C). The cells were plated at a $2 \times 10^4$ cells/well density, in 10% DMEM medium supplemented with 10% fetal bovine serum (Invitrogen, Branchburg, NJ, USA), 100 units mL-1 antibiotic and antimycotic (Sigma Chemical Co.). After 24 hours, 4, 7 and 14 days of culture, the osteogenic parameters were evaluated: cellular viability, and measurement of total proteins and alkaline phosphatase.

### *Analysis of Cellular Adhesion and Viability*

The analysis of cellular adhesion was conducted by counting the number of cells previously adhered using the trypan blue method. The cells were plated and, after waiting for 4 hours for adhesion in a humidified $CO_2$ incubator with temperature control, the supernatant was collected and the wells were washed with a phosphate buffer solution (PBS; Sigma Chemical Co.). Next, the release of cells with trypsin was performed, they were transferred to a 1.5 ml tube, inactivated and counted in a hemocytometer using trypan blue to determine the number of viable cells, non-viable cells, and total cells. Using this method, it was hypothesized that only the adhered cells would remain on the disc after washing it and, therefore, only these were quantified.

After completing the 4 and 24 hour periods, the plates containing the discs were submitted to the cellular viability test using the MTT-formazan colorimetric method (Sigma Chemical Co.), which is based on mitochondrial lactate dehydrogenase activity. 50µl of MTT substrate diluted in a 5 mg/ml concentration of double distilled water was added to each well. Next, the plates were transferred to the $CO_2$ incubator where they remained for 4 hours. After this period, the formazan crystals formed in the reaction were dissolved using 100µL dimethyl sulfoxide (DMSO; Sigma Chemical Co.). Cellular viability was determined by measuring the absorbance using an ELISA reader at 570 nm (Flow Titertek® Multiskan PLUS MK11 - ICN, Finland). The data obtained at 4 and 24 hours were compared. For each experimental time, the results were expressed as the mean values of absorbance, which were used to determine the growth curve.

### *Total Protein and Alkaline Phosphatase Quantification*

After 4, 7 and 14 days of plating, the wells were washed three times with slightly warmed PBS and 2 ml of 0.1% Lauryl Sulfate (Sigma) was placed in each well, then left in ambient temperature for 30 minutes. Aliquots of 1 ml of this solution were withdrawn and placed in test tubes along with 500µl of Lowry Solution (Sigma) for 20 minutes at ambient temperature. Then, 250µl of Folin reagent (Sigma) was added, kept for 30 minutes and read using a spectrophotometer (Biomate 3 - Thermo Scientific, Waltham, MA, USA) at 600 nm. The total protein content was calculated, based on a standard curve and expressed as concentration.

After 4, 7 and 14 days of plating, using the commercial kit (Labtest, Lagoa Santa, MG, Brasil), it was possible to quantify

the alkaline phosphatase activity. The principle behind this kit involves the release of p-nitrophenol and inorganic phosphate from the p-nitrophenyl phosphate in the alkaline medium, a reaction involving the alkaline phosphatase enzyme. Aliquots of the same solution, taken from the wells to quantify the total proteins, were used. These aliquots were added to the contents of the kit according to instructions provided. Absorbance was calculated using the spectrophotometer (Thermo Scientific) at 590 nm and the alkaline phosphatase activity was calculated using a standard curve. The data obtained were calculated by µmol per hour of thymolphthalein and normalized by the number of cells counted after 4, 7 and 14 days.

### *Statistical Analysis*

The data were submitted to analysis for normal distribution and homogeneity of variance (Shapiro-Wilk and Levene's Test). After treating these assumptions, the data were analyzed using one-way ANOVA, followed by the Tukey test. The α=0.05 level of significance was used in all tests.

## RESULT

The mean and standard deviation values for cellular adhesion to the different surfaces are shown in Figure 1. ANOVA showed that there were differences between the surfaces. The Tukey test showed that surface C resulted in significantly greater cellular adhesion than did the surfaces of Exp1 and Exp2, which presented similar values for cellular adhesion.

The values of cellular viability for the periods of 4 and 24 hours for all surfaces are shown in Figure 2. ANOVA showed that there was no significance for the type of surface factor in any of the periods evaluated.

The mean and standard deviation values for total protein for the different surfaces, for the 4, 7 and 14 day periods, are shown in Figure 3. ANOVA showed that there was significance for the surface factor for the 4 and 7 day periods evaluated; however, there was no difference for the 14 day period. For the 4 and 7 day periods, the Exp1 and Exp2 surfaces showed similar levels of total protein and significantly greater levels for surface C. For the 14 day period, the three surfaces show similar values for total protein.

The mean and standard deviation values for phosphatase for the 4, 7 and 14 day periods for the different surfaces are shown in Figure 4. ANOVA showed significance for the surface factor in the three periods evaluated. For the 4 day period, the Exp2 surface showed significantly higher levels of alkaline phosphatase than the surface of Exp1, and is greater than surface C. For the 7 day period, the Exp1 surface showed significantly higher levels than the Exp2 surface, which is higher than surface C. For the 14 day period, the Exp1 surface showed levels similar to surface C and both were higher than the Exp2 surface.

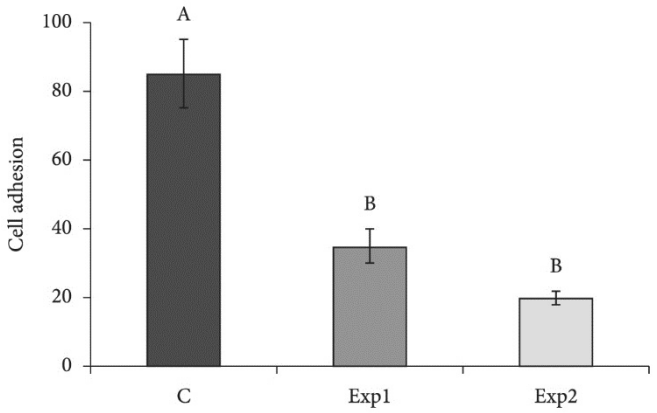


**Figure 1.** Analysis of cellular adhesion, quantified and classified from the percentage of viable cells adhered to the different surfaces in C, EXP1 and EXP2. (Different letters represent significant differences).

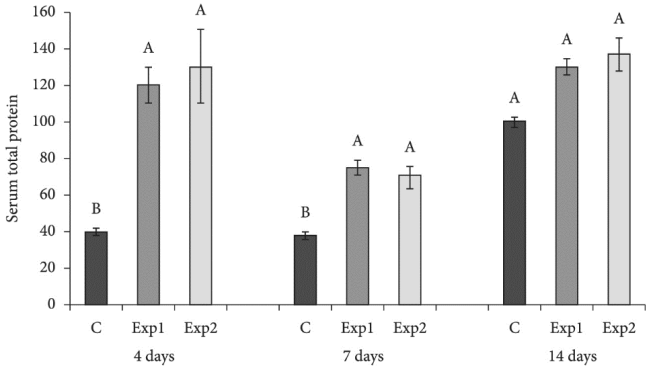


**Figure 3.** Analysis of total protein, quantified and classified as to the mean values of absorbance for each experimental time (4, 7 and 14 days), on the different surfaces in C, EXP1 and EXP2. (Different letters represent significant differences).

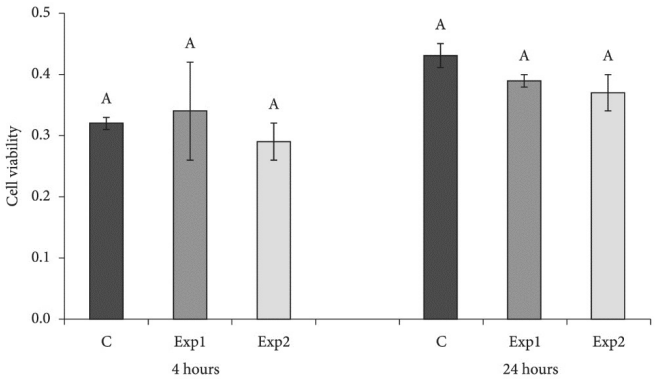


**Figure 2.** Analysis of cellular viability, quantified and classified as to the mean values of absorbance for each experimental time (4 and 24 hours), on the different surfaces in C, EXP1 and EXP2. (Different letters represent significant differences).

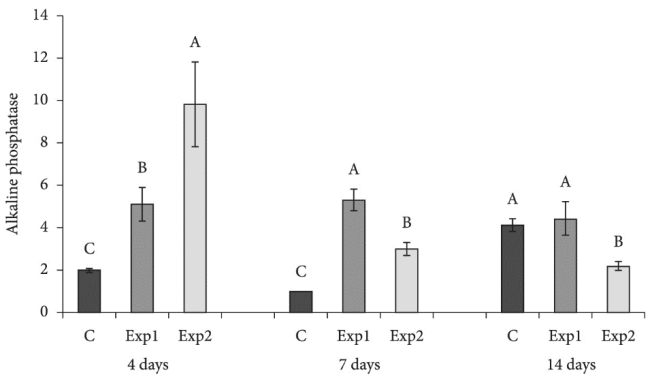


**Figure 4.** Analysis of alkaline phosphatase, quantified and classified as to the mean values of absorbance for each experimental time (4, 7 and 14 days), on the different surfaces in C, EXP1 and EXP2. (Different letters represent significant differences).

## DISCUSSION

The results of the present study suggest that the microstructure of titanium surfaces may affect important parameters of in vitro osteogenesis in the osteogenic cell culture model of the SAOS-2 lineage. Relevant differences were observed in this study of cellular adhesion, as the Control Group showed greater adhesion in relation to Groups Exp1 and Exp2. This fact diverges from some studies[17]. However, it is believed that such divergence occurred due to the use of the titanium discs as control groups in these previous studies, whereas the present study used the polystyrene bottom of the plate, which presents considerable cellular adhesion, for the control group.

In the present study, the results suggest that cellular viability is remained stable in the tests performed with the Control Group, Exp1 and Exp2 after 4 and 24 hours. Sader et al.[11], in 2005, compared surfaces treated with an abrasive system, blasted with aluminum and submitted to acid etching. Cellular viability evaluated after 14 days showed superior viability in the group treated with the abrasive system, while at 28 days a balance could be seen in the number of viable cells in the samples studied.

The blasting and the acid etching represent the treatments of implant surfaces used most when the objective is the creation of surface micro-roughness[11,13,18]. In vitro and in vivo[6] studies compared different types of surface treatments such as blasting, acid etching and acid etching after blasting. The surfaces that showed greater roughness, such as the blasted and blasted after acid etching, promoted the adhesion of osteoblasts in vitro and, consequently, accelerate the process of in vivo osseointegration.

Cellular responses between the bone and the implant depend on characteristics of the surface of the implant itself, such as chemical composition, surface energy and microtopography. These properties determine which proteins will be adsorbed, the amount and the orientation they will have on the surface, thereby influencing mechanisms such as recruitment, adhesion, proliferation and cellular differentiation[6,18]. Thus, it is understood that the surface treatment of titanium also acts indirectly in osteoblast adhesion from the interaction with proteins that will mediate cellular adhesion and differentiation with contribution of proteins from the extracellular matrix, membrane and cytoskeleton[19,20].

In vitro studies with osteoblast cultures reported, from the quantification of total protein, greater expression of the genes involved in the synthesis of proteins in the matrix such as bone sialoprotein, osteopontin, Type I collagen and osteoblast cell proliferation[19,21,22]. In the present study, the analysis of total protein results suggested a significant increase in the rate of absorbance of groups Exp1 and Exp2 in relation to the Control Group. This leads to the idea that the rough titanium surfaces are more effective for the expression of proteins. In addition, it is noticed that when comparing groups Exp1 and Exp2 separately, no significant statistical differences in absorbance are seen and that group Exp2 suggests slightly better numerical results on the fourth and fourteenth days. Therefore, it may be stated that Group Exp2, with greater wettability, would be the group more related to the process of cellular differentiation.

It is believed that osteogenic differentiation occurs sequentially with the appearance of specific osteogenic markers. Therefore, alkaline phosphatase would be the first specific marker that would be linked to osteogenesis, followed by osteopontin and osteocalcin. Several studies have used the presence of alkaline phosphatase to indicate the presence or absence of mineralization on titanium surfaces[17,23,24]. In a study done using chemical acid and heat treatment (control group) and pure chemical acid (test group) on titanium surfaces, alkaline phosphatase activity was analyzed. Thus, the presence of alkaline phosphatase in the group treated with chemical acid was superior to the control group during the two experimental times at 7 and 14 days[18].

## CONCLUSION

Within the methodological limits and from the analysis of the data obtained, the tested surfaces showed differentiated behavior in the quantification of normalized alkaline phosphatase, showing that Exp2 is related to the processes of cellular induction and differentiation; and, that Exp1, treated with blasting and double acid etching, is related to the process of mineralization.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## *CORRESPONDING AUTHOR

Priscilla Barbosa Ferreira Soares, Av. Pará, 1720, Bloco 4L, Anexo A, 38400-902 Uberlândia – MG, Brazil,
e-mail: pbfsoares@yahoo.com.br

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