Brazilian Dental Science

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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

ORIGINAL ARTICLE

DOI: https://doi.org/10.14295/bds.2022.e2528

Influence of titanium nanotubular surfaces, produced by anodization, on the behavior of osteogenic cells: in vitro evaluation

Influência da superfície nanotubular do titânio, produzido por anodização, no comportamento de células osteogênicas: avaliação *in vitro*

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ABSTRACT

Objective: The objective of this study was to evaluate in vitro the influence of the anodized surface of Ti35Nb7Zr alloy on the behavior of osteogenic cells, for future application in biomedical implants. **Material and Methods:** For the development of this research, samples of commercially pure titanium (TiCp) and samples of Ti35Nb7Zr alloy were anodized, both were characterized by scanning electron microscopy (SEM) and were plated afterwards with human osteoblast-like cells (MG63 line) (2×10^4). Cell adhesion, cytotoxicity test, formation of mineralization nodules and a comet assay were also performed in different periods. The bottom of the plate was used as a control, without a sample. **Results:** SEM analysis showed that the topography of both samples presented surfaces covered by nanotubes. Cellular morphology exhibited spreading in both samples proposing an intimate cell-material liaison. After 3 days, the Ti35Nb7Zr group exhibited greater cell viability than the TiCp group ($p<0.01$). Regarding calcium content, there was no statistical difference between the anodized groups, but there was a difference between the experimental groups and the control group ($p<0.01$). In the comet assay, the percentage of DNA in the comet tail did not exhibit any significant difference ($p>0.05$) among the groups in the evaluated periods. **Conclusion:** It was concluded that this process of anodization was efficient to form nanotubes, as well as promote a positive influence on the behavior of osteogenic cells without promoting cell damage.

KEYWORDS

Nanotopography; Osteoblast; Titanium alloy; Titanium implants.

RESUMO

Objetivo: O objetivo deste estudo foi avaliar *in vitro* a influência da superfície anodizada da liga Ti35Nb7Zr no comportamento de células osteogênicas, para futura aplicação em implantes biomédicos. **Material e Métodos:** Para o desenvolvimento desta pesquisa, amostras de titânio comercialmente puro (TiCp) e amostras da liga Ti35Nb7Zr foram anodizadas, ambas foram caracterizadas por microscopia eletrônica de varredura (MEV) e posteriormente plaqueadas com células semelhantes a osteoblastos humanos (linha MG63) (2×10^4). Foram realizados em diferentes períodos a adesão celular, teste de citotoxicidade, formação de nódulos de mineralização e ensaio do cometa. O fundo da placa foi usado como controle, sem amostra. **Resultados:** A análise em MEV mostrou que a topografia de ambas as amostras apresentava superfícies cobertas por nanotubos. A morfologia celular exibiu espalhamento em ambas as amostras, propondo uma ligação íntima célula-material. Após 3 dias, o grupo Ti35Nb7Zr exibiu maior viabilidade celular do que o grupo TiCp ($p<0.01$). Em relação ao teor de cálcio, não houve diferença estatística entre os grupos anodizados, mas houve diferença entre os grupos experimentais

e o grupo controle (p <0.01). No ensaio do cometa, a porcentagem de DNA na cauda do cometa não apresentou diferença significativa (p> 0.05) entre os grupos nos períodos avaliados. **Conclusão:** Concluiu-se que esse processo de anodização foi eficiente para formar nanotubos, além de promover uma influência positiva no comportamento das células osteogênicas sem promover dano celular.

PALAVRAS-CHAVE

Nanotopografia; Osteoblastos; Liga de titânio; Implantes de titânio.

INTRODUCTION

In an attempt to reduce or even eliminate the period of bone healing, providing a better quality of life and longevity to patients, a wide variety of materials, natural and synthetic have been developed in recent decades. The interest of researchers was increasingly aroused with regard to the development of new biomaterials that direct and enable osseointegration more quickly for application in the orthopedic and dental areas [1-6].

Of major interest in the field of biomaterials studies are titanium alloys, among them, the titanium-niobium-zirconium alloy (TiNbZr) has been the target of several studies due to biocompatibility and physicochemical properties such as: chemical inertness; excellent ductility; high melting point; high capacitance; excellent conduction of heat and electricity; and low modulus of elasticity [6-9].

In addition to the characteristics of the biomaterials from which the implant was produced, the characteristics of the implant surface are considered relevant due to their great influence on the quality of the osseointegration obtained [10,11], since they influence the process of activation, differentiation and proliferation of cells with osteogenic potential, which will reflect in bone morphogenesis [3,12-15].

Nanotechnology is the application of science and engineering on an atomic scale, which facilitates the construction of new materials and devices by manipulating individual atoms and molecules and, moreover, allows the atom-by-atom construction of tiny structures (1-100 nm) [16-18], which have new properties and major applications in the health sciences and biotechnology [19]. Nanotubes have properties such as high electrical conductivity, high chemical stability and high mechanical resistance, which makes them potentially interesting for biomedical applications, as the configurations and physicochemical properties of nanostructured materials influence cellular interactions that lead to tissue regeneration, so due to this they are considered an advance on implantable surfaces [20].

Modifications on the surface of implants, on a nanometric scale have been proposed in order to improve the biological properties that involve the bone-implant interaction [1,3]. Anodic oxidation is one of these alteration methods and consists of an electrochemical surface treatment process, which allows preserving all the qualities of the metal, protecting it from the aggressiveness of the environment, by creating an oxide layer on the surface. The new oxide layer is firmly adhered to the substrate, which benefits the biological response of the implant and gives the method an advantage. The growth of organized nanotubular oxide structures on implant surfaces can directly affect cell behavior when these materials are used in biomedical applications, enabling cell proliferation, leading to increased osteoblast adhesion and bone neoformation [21-23].

Since nanostructured surfaces can modulate and accelerate the cellular response and that TiNbZr alloy presents low elastic modulus and is promising as a biomaterial, this study is being proposed to evaluate cellular behavior on Ti35Nb7Zr samples, with growth of nanotubes on their surface by means of cellular cytotoxicity (MTT), formation of mineralization nodules, quantification of calcium content, and evaluation of genotoxicity by the comet assay. With the association of these results, it is estimated that the in vitro biocompatibility of the nanotubes produced in the Ti35Nb7Zr alloy is proven, and to estimate the possible reduction of the osseointegration period due to the cell-implant interaction in the search for an ideal biomaterial for biomedical use.

Therefore, this study aimed to evaluate in vitro the influence of the anodized surface of Ti35Nb7Zr alloy on the behavior of osteogenic cells (human osteoblast-like cells - MG63 line), for application in biomedical implants.

MATERIALS AND METHODS

Anodizing process

Commercially pure titanium (TiCp) samples, and Ti35Nb7Zr alloy samples were subjected to the anodizing process. Both samples used in the present study and the anodizing process were performed in the Department of Engineering Materials (DEMAR) – Lorena Engineering School (EEL) - USP.

The Ti35Nb7Zr alloy was produced by arc furnace fusion with a non-consumable tungsten electrode in an argon atmosphere (99% purity) in a water-cooled copper crucible. Initially, high purity sheets of the zirconium (99.5%), niobium (99.8%) and pure Ti grade IV (CpTi) (99.5%) were purchased (Muiller Ltda, Sao Paulo, Brazil), which were cut by means of a guillotine into strips appropriate to fit the size of the crucible and then pickled in acid solution suitable for each metal. The clean materials were weighed in proportions suitable to obtain the samples. The materials were vacuum encapsulated in quartz tubes and then subjected to solubilization at 1000ºC for 2 hours (1000ºC / 2hrs. WQ), cooled in water and then recrystallized at 700ºC for 30 minutes (700ºC / 30mins. WQ),and cooled in water.

Next, the anodizing process was performed using a solution composed of glycerin (Synth), 50% (v/v) deionized water, and 1% by mass of ammonium fluoride (NH_4F). It was subjected to a voltage of 20 V and 3 A for a period of 2 hours.

The disk samples, measuring 6X4 mm, were kept in a desiccator until the time of characterization and biological evaluation. Prior to cell culture, the samples were characterized by Scanning Electron Microscopy (SEM) with Field Emission (FEG-SEM Zeiss Supra 5VP) from National Synchrotron Light Laboratory (LNLS) in Campinas.

Cell culture and in vitro assays

The culture of human osteoblast-like cells (MG63 line) was obtained from the Paul Ehrlich Association of Scientific Technical Banks (MG-63, APABCAM, Rio de Janeiro, Brazil). The MG63 cells were cultured in 75 cm^2 flasks (TPP, Biosystems, Curitiba, Brazil), humidified atmosphere (CO_2, 95%, 37°C) in DMEM medium (Gibco) with 10% FBS (LGC Tenchology, Campinas Brasil), 100 U mL^{-1} of penicillin (Gibco-Life Technologies, NY, USA). and 100 μg^1 streptomycin (Gibco- Life Technologies, NY, USA).

After confluent monolayer formation (about 07 days), the cells were removed using a solution containing 0.25% trypsin (Cultilab Ltda, Campinas, Brasil) and counted for plating (Cell Counter Countess®, Invitrogen, USA). Before the experiment, all samples were sterilized under a UV lamp for 30 mins. For all assays 2 x 10^4 cells/ well were placed in 24-well polystyrene plates (TTP, Biosystems, Curitiba, Brazil) with the samples previously inserted. For the control group the cells were grown in 24-well polystyrene plates without samples. For in vitro assays, the disks were removed from the original plates and transferred to other clean plates, and during the plating, the old medium was removed every three days.

For evaluation of cell viability, the cells were cultured in the samples at a concentration of $2x10^4$ viable cells/well for 3 days using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma) solution. The cells were incubated with MTT solution at 37°C for 4 hours; this assay was performed as described by Rosa et al. [11]. After, the supernatant was removed, the samples were washed with PBS, and 1 mL of isopropanol acid (0.04 HCl in isopropanol) was added to each well. Colorimetric analysis was performed with an EL808IU spectrophotometer (Biotek Instruments, Winooski, VT, USA) at 650-570 nm. Cell viability data was normalized using the control group and expressed as percentage.

After 14 days in culture, a bone-like mineralized nodule formation was accessed using staining with 2% red Alizarin S (Sigma). Initially, the samples were washed 3 times with Hanks solution (Sigma) at room temperature, and the cells were fixed with 70% ethanol for 1 hour at 4°C.Then the samples were washed again with PBS solution and finally stained with red Alizarin pH 4.2, for 15 minutes at 37°C. Afterwards, the samples were washed with deionized water and dried for 24 hours at room temperature. The quantification of Alizarin red staining as calcium content was performed according to the method previously described by Gregory et al. [24], using 560 μL of 10% acetic acid in each well, and after 30 minutes, 40 μL of 10% ammonia hydroxide was added. The results were read at 405 nm in a spectrophotometer. Values were expressed as absorbance.

The human osteoblast-like cells were cultured for 3 and 7 days in cell culture flasks with the culture medium, which remained in direct contact with the samples. In the pre-determined periods, the comet assay was performed, using the cells (1×10^5) suspended in 200 μL of 0.5% low melting point agarose. The cells were transferred to newly prepared slides with 1.5% agarose (the slides were prepared with one agarose layer). After polymerization of the layers the slides were placed in a 0.3 mol/ L electrophoresis buffer (NaOH, 1 m M/ L, Na_2EDTA, pH 13) and electrophoresed at 300 mA and 1.0 V/ cm for 20 mins. The slides were neutralized with TRIS-HCI (pH 7.5) and stained with ethidium bromide (20 μg/ml) for 10 min. All steps of the analysis were performed without direct light to avoid further damage to DNA. The images were acquired with a Leica DMLI epifluorescence microscope. The comet images were automatically evaluated with OpenComet analysis software [25]. The extent of DNA damage was expressed as a measure of the percentage of DNA in the comet tail.

Statistical analysis

Statistical analysis was performed using parametric or nonparametric tests, for independent data (ANOVA or Kruskal-Wallis, respectively), and followed by a multiple comparison test when necessary (Tukey and Dunn, respectively). The level of significance was 5%. Statistical analysis as performed using GraphPad Prism software (GraphPad Inc., La Jolla, CA, USA).

RESULTS

Analysis by scanning eletron microscopy

Figure 1 show the images of TiCp and Ti35Nb7Zr alloy anodized samples, respectively. In these figures the topography of the samples were observed in longitudinal view, which presented nanotubes with similar patterns, ordered perpendicular to the surface.

Cell adhesion

SEM analysis was also performed within 24 hours after culturing cells to study the morphology of the osteogenic cells in contact with TiCp and Ti35Nb7Zr (Figure 2). Cellular morphology showed spreading in the two samples, suggesting an intimate cell-material contact relation.

Cytotoxicity assay

Cell viability results of the materials is shown in Figure 3. After 3 days, the Ti35Nb7Zr group exhibited higher cell viability than the TiCp group ($p<0.01$), but the Ti35Nb7Zr group showed viable cell percentage similar to the control group ($p>0.05$). The results of the cytotoxicity assay are shown in Figure 3a.

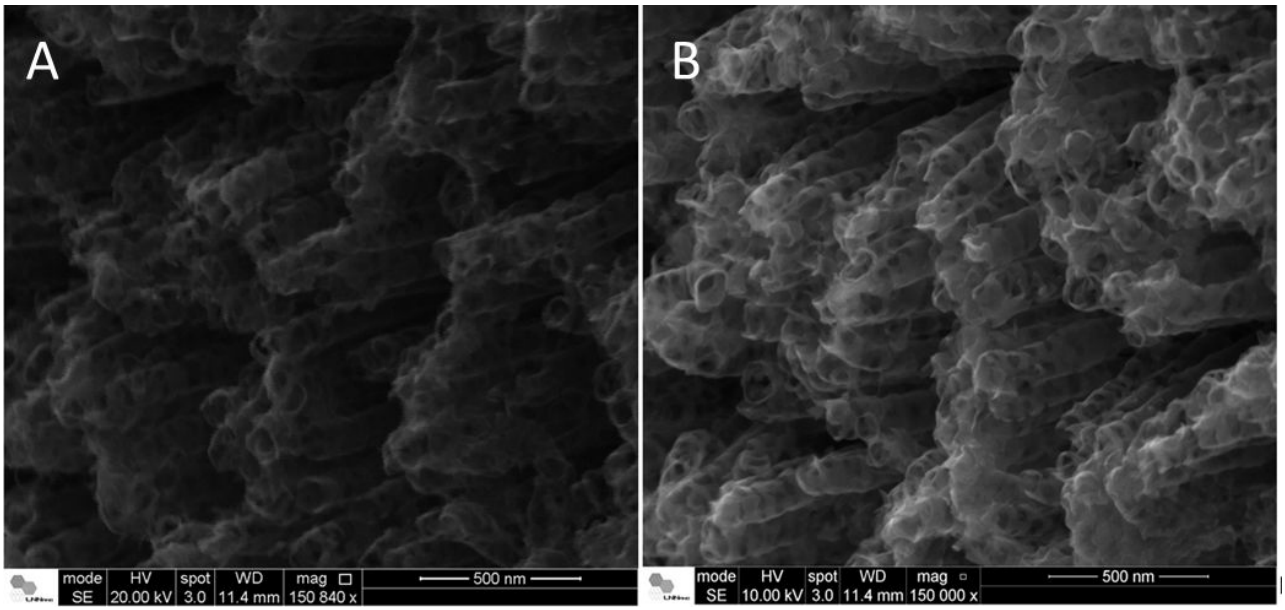

Figure 1 - SEM - FEG photomicrographs (scale bar 500 nm) showing the morphology of anodized samples: (A) TiCp sample; (B) Ti35Nb7Zr sample. Note the nanotubes in longitudinal view.

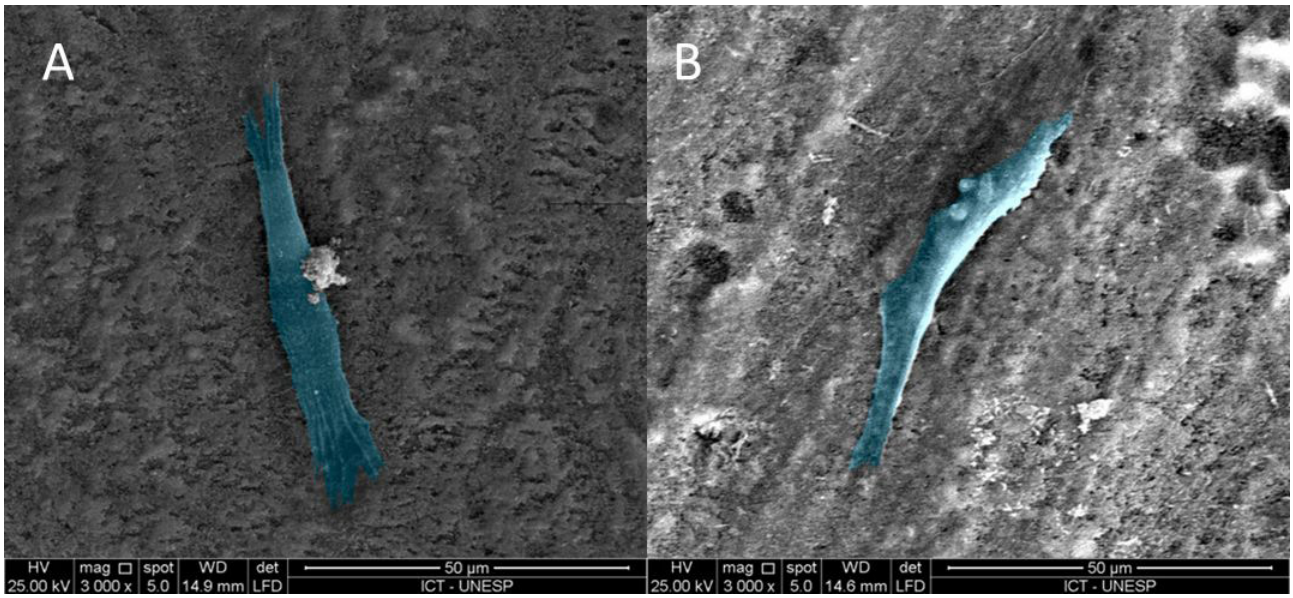

Figure 2 - SEM - FEG photomicrographs showing the morphology of osteogenic cells on the sample's surface after anodization: (A) surface of a TiCp sample; (B) surface of a Ti35Nb7Zr sample. Note the filopodial extensions formed on the samples (scale bar= 50µm).

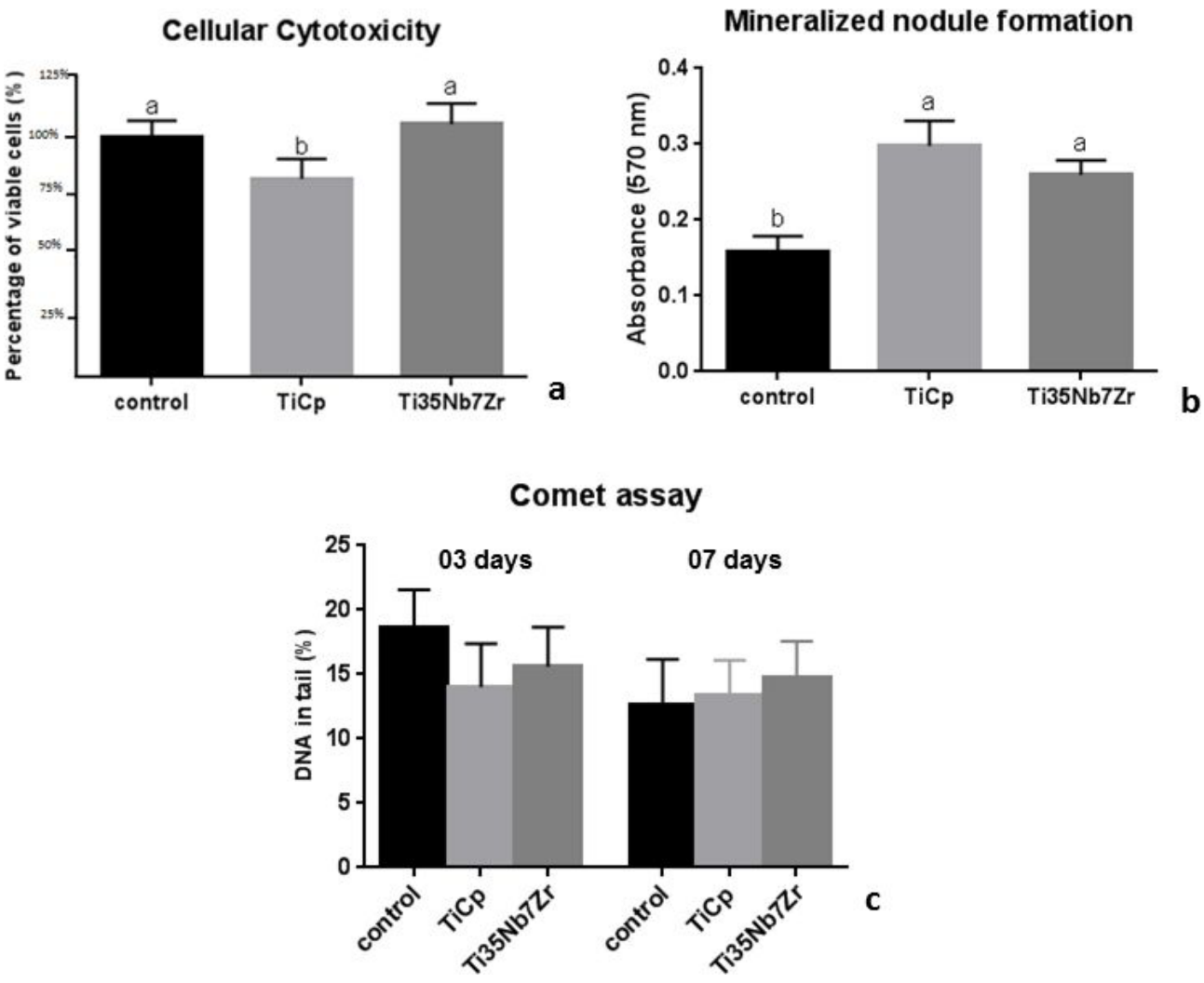

Figure 3 - Representative graph of the effect of surface topography on osteogenic cell behavior: (a) percentage of cellular viability; (b) quantification of calcium content by alizarin red staining b; (c) percentage of DNA in the tail in the comet assay. Different letters indicate a statistical difference ($p<0.05$).

Mineralization nodule

Calcium content stained by Alizarin red not after 14 days cell culture was not affected by the composition of material, because anodized samples did not show any statistically significant difference between them ($p>0.05$), but a difference with the control group ($p<0.05$) was observed. Data is presented in Figure 3b.

Comet assay

At 03 and 07 days, the percentage of DNA in the tail of the comet was not significantly different between the groups, $p= 0.5026$ and $p= 0.4280$, respectively, in the Kruskal-Wallis test (Figure 3c).

DISCUSSION

Titanium (Ti) is the most used material in conditions where high performance and reliability is required due to its intrinsic properties of: high mechanical strength/ weight ratio; low specific mass; high corrosion resistance; excellent biocompatibility; the property can be forged by conventional techniques and be cast using precision casting techniques and can be processed by powder metallurgy [11,26]. These properties of titanium make it and Ti alloys the most frequently used metallic materials in aerospace, chemical, naval and implantological industries [27]. However, implants produced from commercially pure titanium and used in clinical, orthopedic and dental practice, have a drawback, which is the difference between the elastic modulus of the Ti – 110 GPa metal implant and the modulus of elasticity of the bone – 10 – 35 GPa [28-30]. However, this value of elasticity can be controlled in two ways: for the manufacture of implants using Ti alloys [26,27,31,32]; or for the creation of porosities in the implants [7,33,34]. These alternatives aim to reduce the difference between the modulus of elasticity of the materials. In addition, the combination of both can be used for the production of Ti alloy implants with porosity [8,35]. Thus, a better outcome in the performance of the bone-implant system is expected. In this context, the composition of the materials and the surface topography demonstrate a great influence on cellular activity as previously demonstrated by Qadir et al. [1]; Mello et al. [5]; Sista et al. [14,15], and in this study. The cell line used (human osteoblast-like cells - MG63 line) was based on International Standards Organization ISO 10993-5 guidelines [36] and previous studies [4,5,12,37-39].

Titanium-niobium-zirconium alloy (TiNbZr) exhibits an adequate elastic modulus and high mechanical and corrosion resistance [9,34]. Additionally, it was shown to be osteoconductive and biocompatible in vivo, non-cytotoxic in vitro, and there are no reports on its mutagenicity or carcinogenicity action [2,7,8,31,40,41]. Recently Mello et al., 2019 [5] demonstrated that the nitric oxide released by cells when in contact with Ti13Nb13Zr alloy was similar to the value released by the titanium used commercially. The authors also reported the satisfactory properties of this alloy in relation to proprieties of this alloy in relation to antimicrobial, antibiofilm, and osteogenesis activities.

Physical-chemical treatments of the Ti surface have been proposed with the purpose of improving the biological performance of the implants, by obtaining nanotopography on the surface. The interest in obtaining nanotopography through the anodizing process has been increasing, since this technique exhibits adequate surface modification, with pore production and stabilization of the oxide layer, resulting in a positive effect on cellular activities [3,9,13,14,23,32,42-44]. Surface chemical modification procedures have provided an alternative or additional method for physical-chemical and topographical changes of materials. The chemical surface modifications make use of the current knowledge of biology and cellular biochemistry to improve the function and differentiation of the cells, besides optimizing the mechanisms of cellular adhesion to the substrate [12,13]. The aim of these surface alterations is to immobilize proteins, enzymes or peptides on the biomaterials, aiming specifically to induce cellular and tissue responses. Mello et al. [6] evaluated the basic elements presents in the Ti35Nb7Zr and concluded that Zr has a good influence on bone marrow stromal cell differentiation.

Anodizing is one of the methods used to create nanotopography and improve interfacial properties. This method has increased the life span of implants and has attracted great attention from researchers [13,14,21,42,43]. There are many techniques for anodization using different acids such as H_2O_2 [42], H_3PO_4 [13,34], HF [14],

H_2SO_4 [23] with various voltages; furthermore, anodization can be performed in single or multiple steps [21,42]. In the present study, the acid used was NH_4F in a single step. All these methodologies were efficient in promoting nanotubes, as in our study. These treatments are specific electrochemical processes capable of forming a more predominant oxide layer in the anatase phase, which is known to have an electrical conductivity greater than that of amorphous TiO_2, favoring surface bioactivity and also charge transfer [13]. Additionally, anodization promotes different thicknesses and surface porosities of TiO_2 films on titanium and its alloys that appear to have a different behavior combination of bioactivity, chemical stability and mechanical integrity [15,23].

Surfaces modified by the anodization technique are being proposed to promote interactions of the chemical composition of the implants with the tissues and cells. Therefore, the bioactivity of materials that received the anodizing technique is improved when compared to metals that do not receive the same process [3,12,14]. Moreover, studies have demonstrated cell adhesion and orientation, as well as more accelerated osseointegration on these anodized surfaces [13,21,42-44]. Our study also demonstrated that the anodized surface, under the parameters described, allows a large number of viable cells when evaluated in the short term, and cell-adhesion was observed on anodized samples with spread cells that suggest adequate cell-material contact. The calcium content stained by Alizarin red formed on the anodized surface of the TiCp and Ti35Nb7Zr alloy did not show any difference between them. However, the anodized samples, independently of composition, induced greater calcium content than the control group, with a statistical difference. Alizarin red staining is an indicator of calcium production by mature osteoblastic cells [45]. Higher calcium concentrations with the formation of mineralized nodules were found in the groups with a nanotubular surface in relation to the non-anodized group. In all the tests, it was possible to observe the positive influence of the samples' surface topography with nanostructured oxide layers and, as before, an enhanced response from crystalline layers. This data indicates that this alloy, when subjected to the anodizing process, can be used as biomaterial.

Previous studies have demonstrated the genotoxicity effect caused by biomaterials, through the comet assay, which individually evaluates cell DNA damage under different experimental conditions [46]. It is a rapid and sensitive technique [47]. The results of the present study showed that in both evaluated periods, there was no induction of DNA fragmentation in the cells exposed to the different samples.

This study evaluated the behavior of osteogenic cells in contact with anodized Ti and Ti35Nb7Zr alloy samples by means of the cell adhesion and cytotoxicity test, quantification of mineralization nodules and genotoxicity analysis. The surface of the anodized samples was evaluated by means of SEM that demonstrated the presence of nanotubes distributed throughout the surface. The results of the in vitro tests showed that the anodizing parameter is biocompatible, since the experimental samples presented similar influence in their cellular behavior when compared to the anodized surface of Ti that was used as a control. These results suggest that this alloy, due to its better mechanical properties, may be a promising biomaterial for the manufacture of surgical implants aiming at biomedical application.

CONCLUSION

Based on the results, it was observed that these parameters of the anodization technique are efficient in forming nanotubes on the surfaces of TiCp and TI35Nb7Zr alloys, and these anodized surfaces promote a positive influence on the behavior of osteogenic cells without promoting damage cell, and can be considered for biomedical applications.

Acknowledgments

The authors would like to thank the the Coordination for the Improvement of Higher Education Personnel (CAPES) for for financial support for the scholarship.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

The authors declare that no financial support was received.

Regulatory Statement

The authors declare that this work does not require the approval of the ethics committee.

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Date submitted: 2020 Sept 24
Accept submission: 2021 May 18