Evaluation of annealed titanium oxide nanotubes on titanium.From surface characterization to *in vivo* assays

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Abstract

The entire route from anodic oxidation and surface characterization, including *in vitro* experiments and finally *in vivo* osseointegration assays were performed with the aim to evaluate nanotubular and crystalline annealed titanium oxides as asuitable surface for grade 2 titanium permanent implants.

Polished titanium (T0) was compared with anodized surfaces obtained in acidic media with fluoride, leading to an ordered nanotubular structure of titanium oxide on the metal surface, characterized by tube diameter of 89 ± 24 nm (Tnts). Samples were thermally treated in air (TntsTT) to increase the anatasecrystalline phase on nanotubes, with minor alteration of the structure. Corrosion tests were performed to evaluate the electrochemical response after 1, 14 and 28 days of immersion in simulated body fluid (SBF). Based on the *in vitro*results, heat treated titanium nanotubes (TntsTT) were selected as a promissory candidate to continue with the osseointegration*in vivo* assays.The *in vivo* results showed no major improvement in the osseointegration process when compared with untreated Ti after 30 days of implantation and there also was a lower increase in the development of new osseous tissue.

Keywords:

Titanium nanotubes-Anodization-Thermal treatment-Osseointegration

1. Introduction

A rapidly established, strong and long-lasting connection between an implant and bone is essential for the clinical success of orthopedics and dental implants. Metal implants are preferred for load-bearing applications because they exhibit excellent mechanical properties such as young´s modulus, tensile strength, ductility, fatigue life, and wear resistance[1,2].Requirements for permanent metallic implants include properties such as corrosion resistance, and biocompatibility. After decades of study and improvement of metallic implant quality, the influence of the surfacecharacteristics of the devices in osseointegration process is indubitable[3,4]. Surface modification strategies induce changes in

physico-chemical properties or alter the architecture of the surface topography[5]. Most processes induce both type of changes simultaneously over different length scales[6,7].

The surface technology of metallic implants have considerably progressedfrom bioinerts surfaces to bioactive surfaces and further to biomimetic engineerednano-texturized surfaces such as titanium coveredwith TiO_2nanotube arrays[7]. Some works suggested that nano-surface characteristicsincrease the rate of initial osseointegration between Ti alloys and the surrounding tissue, strengthening the bond between implants and new bonegrowth[8–10].

Titanium anodic oxides growth may lead to different structures depending on the acid mixtures used as electrolyte[11–13]. The anodic oxide structure is characterized by the presence of nanotubes when fluorides are included in the electrolytic media[14]. Fluoride ions are responsible for the formation of nanotubes or pores in the titanium oxide in a growth-dissolution process during anodization[15–17].

Other crucial aspect of surface titanium oxides as promoter of osseointegrationare crystalline phases. Anodized nanotubes are often amorphous[11]and post anodization treatments are performed to obtain anatase, rutile or mixture of both phases[18,19]. Thermal treatments are currently the most effective route to obtain crystalline nanotubes.Anatase is pointed as the higher bioactive phase[18,20]. The crystalline phases may differ after thermal treatments for nanotubes with different diameter[21].

In this work, nanotubes were produced by anodic oxidation in commercially puretitanium (CP Ti), controlling the structural features of anodic oxides, with anatase as a bioactive crystalline phase and studying the impact on new bone formation rate.The morphology, crystalline phases and electrochemical behaviourwere characterized in a simulated body solution (SBF). Finally,*in vivo*experiments in a rat model were performedto evaluate the characteristics of the new bone in closed contact with Ti implants.

2. Experimental

2.1. Materials and surface modification treatments

Flat specimens of 20 x 25 x 0.4 mmof CP Tigrade 2 Ti (wt.%: 99.2, C 0.1 max, Fe 0.3 max, H 0.01 max, N 0.03 max and O 0.25 max, Roberto Cordes, Argentine) were usedfor surface

characterization and electrochemical tests, whereas 0.8 mm diameter x 10 mm length cylindersof the same material were used as implants for *in vivo* assays.

The specimens were surface treated with the conditions detailed in table I. Anodizing process was performed in a twoelectrode cell using a power source Consort EV231 (Belgium) and Pt wire as counter electrode. The electrolytic solution was prepared by dilution of concentrated acids (Sigma Aldrich) with de-ionized water (18.2 MΩcm, Millipore). A Cu wire on an extreme of the samples was used as electrical contact, conveniently isolated from the electrolyte. Before and after each test the samples were cleaned with acetone, dried in air and stored in a dryer.

2.2. Surface characterization

The overall surface morphology of the specimens was observed by scanning electron microscopy (SEM) using a FE-SEM Carl Zeiss Sigma (Germany)operatedin secondary electron modeat 3 kV. Crystalline phases were determined with X-ray diffraction at grazing angle (G-DRX) (PANalyticalX'PertProdiffractometer, UK) and Raman spectroscopy (Invia Reflex confocal Raman microprobe, Renishaw, UK). G-XRD spectra were obtained with Cu-Kα radiation at 40 kV and 40mA, between 10º-70º degrees with $0.02^{o}s^{-1}$ speed at 2º incidence angle. Raman spectra were obtained using an argon laser of 514 nm and a 50x objective lens. No thermal effects were observed on the samples during the measurements.

2.3.Nanotubes diameter Size distribution

Quantitative morphological data such as nanotubes size and size distribution were acquired by SEM micrograph image processing.Image analysis techniques have been widely used for particle size measurement[22]. In this work, nanotubes size distribution throughout the sample was using an automatic algorithm that allows the detection and measurement of the nanotubes diameters by applying three techniques: a) enhancement, b) binarization, and c) characterization by shape factors (maximum diameter). Finally, the histogram corresponding to the size distribution is obtained. The nanotubes size distribution is defined as the discrete function p(dk)=nk, (dk is the kth diameter measured and nk is the number of particles with that diameter).

2.4. Immersion in simulated body fluid solution (SBF)

Electrochemical and immersion tests were performed in a solution with ion concentration similar to blood plasma[23]. All reagents were provided by Sigma-Aldrich (analytical grade, 85.0%).Deionized water (18.2 MΩcm, Millipore) was used throughout. The solution was buffered to pH 7.4 with concentrated HCl and tris(hydroxymethyl)aminomethane (tris).

Titanium samples were maintained in SBF following the recommendations of ISO 23317:20014 (E) standard[24]. Separated sets of specimens were kept in SBF solution for 1, 14 and 28 days at a constant temperature of 37ºC. The sample area (in mm^2) to the solution volume (in mL) ratio was set equal to 10.

2.5. Electrochemical tests

Electrochemical essays were carried out in a Reference600 (Gamry,USA) electrochemical unit ina conventional three electrode cell with saturated calomel electrode (SCE, Radiometer Copenhagen) as reference and a Pt wire as counter electrode (CE). Potentiodynamic polarization curves were conducted from the corrosion potential (Eoc) to 1.5 V and backwards, or up to a maximum current density of 0.001 $A.cm^{-2}$, at a sweep rate of $0.001V.s^{-1}$. Electrochemical impedance spectroscopy (EIS) tests were performed with a perturbation signal of 10 mV rms around Eoc and measured between 10^{-2} and 10^{6}Hz. Prior to every test, the system was mantained at least for 40 minutes up to stabilization. All electrochemical tests were conducted on different samples at least for quadruplicated after 1, 14 and 28 days of immersion in SBF solution maintained at 37°C in stove.

2.6. *In vivo* studies

2.6.1. Animals

Twelve-week-old male WKAH/Hok rats weighing 300-330 g were used. The animals were divided in two groups:Control Titanium (T0) and annealed titanium oxide nanotubes (TntsTT). All animals were housed in a temperature controlled room with 12h of alternating light-dark cycle and provided with water and food *ad libitum* throughout the study. The

experiments were approved by the Bioethics Committee HIEMI-HIGA, (Mar del Plata, Argentine, October2011).

2.6.2. Surgical procedure

The protocol followed for implantation surgery was described elsewhere[25,26].Implants were placed bilaterally ensuing 4implants per rat. T0 andTntsTT implants were placed by press-fit into bothtibia and femur extending into the medullar canal.

2.6.3 Bone labeling with fluorochromes

A polychrome sequential fluorescent labelling method was used to characterize the new bone formation and mineralization process. Different fluorochromes were intraperitoneally administered at a sequence of 30mg/kg alizarin complexone (Sigma-Aldrich, USA),and 20mg/kg calcein (Sigma-Aldrich, USA)after 2 and 4 weeks from surgery.Fluorochromes bind to calcium ions and later can be incorporated at mineralization sites. The animals were sacrificed 30 days after implantation. The fluorescent dyes were detected in histological sections with afluorescence microscope (Nikon EclipseTi, Japan) giving an indication of new extracellular matrix deposition[27].

2.6.4 Histological analysis

Thirty days after implantation, sixrats with T0 and six rats with TntsTT implants (individuals from different litters) were deeply anesthetized with Ketamine/Xylasine (80mg/kg, 10mg/kg) and sacrificed with an overdose of sodium pentobarbital. The retrieved samples were treated as in previous works[25,26].Proximal cross-sections transversal to the central long axis of the tibia and femur (n=6 /surface treatment group) were prepared and cut. Three sections from the proximal regions were selected for this study. Samples were stained with Toluidine blue, that allows the observation and identificationof tissues (e.g. bone and bone marrow) around the metal-implant interface without removing the PMMA and then examined in an optic microscope (Nikon Eclipse Ti, Tokyo, Japan).

Histomorphometricdetermination of the thickness of the newly formed bone in contact with the implants was performed using the software ImageJ (opensource:http

://rsb.info.nih.gov/ij/features.html). The area of the primary press-fit contact of the implant and bone was excluded from the analysis. Thus, only new bone was analyzed[28].

2.6.5 Mineral apposition rate (MAR)

The mineral apposition rate (MAR, μm/day) is the rate at which mineral accretion occurs at a remodeling site during the period of bone formation. MAR is a fundamental histomorphometric variable, and it is a reliable measure of osteoblast function. Fluorochrome sequential labelling was performed postoperatively as reported previously[29,30] (fluorochromes and dosage informed in 2.6.3). The names and symbols used are those describedin conventional bone histomorphometry[31].

2.6.6. Statistical analysis of *in vivo* results

In this study, the data are shown as mean value ± σ_{MEAN} (standard deviation of the mean). Differences between the groups were assessed by a non-parametric test. Man-Whitney test was performed using GraphPad In Stat version 3.00 (Graph Pad Software). All statistical analysis was considered significant when p value < 0.05.

3. Results

3.1. Surface Characterization

3.1.1. Morphology of the oxide films formed on titanium

SEM sequence of the surface appearance of titanium in the three surface conditionsis presented in Fig. 1. Low and high magnification images were taken not only to determine nanometric parameters as nanotube diameter but also to characterize the general aspect of the entire surface. This is not often included in the papers even when the size of biomedical implants are devices with several square centimeters long, and the entire device surface is required to be attached to bone. The main surface characteristics of T0 samples (Fig. 1a-c) is the presence of regular machining groovesand ridges (G-R) and tearing (T) originated in the sheet processing and mechanical polishing[32]. Fig. 1d-freveal the general appearance of the anodized surface (Tnts samples). The fullcoverage of the surface by anodization is evident in low magnification micrographs. In Fig. 1e,the presence of two main featuresat the

micrometrical scale is evidenced, a granulated continuous film and some homogeneously distributed columns, with a characteristic rosette like structure. These rosettes were previously reported as a characteristic shape of titanium anodic oxide growing in acidic media with and without fluoride ions[33,34]. At higher magnifications (Fig. 1f) nanotubes with open (OTnts)and close top (CTnts)are distinguished.Two height levels are well differentiated,the upper correspond to nanotubestop closed whereas the lower is composed for open nanotubes. Finally, TntsTT samples are showed in Fig.1g-i. No major changes in surface features distribution or size due to the heat treatment are detected with SEM analysis when comparing to Tntscondition. No collapse of the nanotubes is observed after heat treatment.

3.1.2. Size distributions of nanotube diameter

Fig. 2 illustrates the algorithm used to determine the nanotube diameters.First, the inner part of open nanotubes was enhanced by a contrast stretching algorithm[35,36].Fig. 2a shows the result as agrayscale image, where enhanced regions represent the inside of the open nanotubes (Fig. 2b). This image shows a bimodal histogram used to determine a threshold value to discriminate the inside nanotubes from the background. In the second step, a binary image isobtained (Fig. 2c).Finally, the maximum and minimum diameters of every object (inside nanotubes) present in the binary image aremeasured according to the magnification of the original image (Fig. 2d). Considering the maximum diameter obtained for every object in the micrograph, the histogram corresponding to the size distribution of nanotubes is obtained (Fig. 2e). The process is repeated with images from different samples with the same surface condition and the results are condensed in Fig. 2f. The size distribution corresponds to a normal distribution with a mean diameter of 89 nm and standard deviation of 24 nm.The same procedure is performed to a set of images of TntsTT samples, and the corresponding histogram is presented in Fig. 2g. In this case, the obtained values are 82 nm with standard deviation of 33 nm.

3.1.2. Crystalline phases – G-DRX and Raman spectroscopy analysis

Fig. 3 shows the G-DRX spectra of the studiedconditions. As expected, T0 spectrum only presents diffraction peaks corresponding to metallic titanium[37]. On anodized samples, the

main peak of anatase (PDF 00-001-0562) appears after heat treatment (TntsTT) whileTnts samples do not evidence crystalline TiO_2 diffraction peaks. X-ray diffraction requires an important amount of crystalline phase present to be detected from diffraction peaks. Even when low incident angle is used, reducing the penetration, metallic peaks appear very intense in anodized samples, due to the small thickness and porosity of the anodic layer compared to the penetration length of the technique. On the contrary, Raman spectroscopy gives information from a few nanometers in depth of the sample surface, and therefore is more sensitive to the presence of small regions of crystalline order in thin films[38].

Fig 4 shows the Raman spectra of the samples. T0 does not present peaks of any crystalline structure, meanwhile Tnts and TntsTTpresent peaks characteristic of crystalline phases insurface film oxide samples[39,40]. Thespectra were taken in different sites over one unique sample. In Tnts, broad, low intensity peaks are obtained in somesites of the sample, expressing the partial crystallinity, small crystal size and a prevalence of amorphous order in the film[41].However, intense anatase peaks are also present. TntsTT spectra showsthat after heat treatment, anatase presence is found in all the sites tested over the sample surface, the intensity of the peaks increased, and rutile is detected as a broad low intensity shoulder.

3.2.Electrochemical behavior in SBF solution

Fig. 5.a. presents the anodic polarization curves after 1 day of immersion in SBF for all the surface conditions studied. T0 presents a continuous increase in the current density with anodic potential until a passive state is reached at 300 mV *vs*SCE. Further anodic polarization does not lead to an increase in current density in the wide anodic potential range recorded, evidencing the passivity of the surface. The porous nanotubular structure in Tnts presents a similar current density than T0 with higher passivation potential, whereas TntsTT samples present an improvement ofthe electrochemical resistance, behaving as a passive surface in all the applied potential range, with a current density around one order of magnitude lower than T0.

Along with anodic polarization curves, EIS gives information of the protective effectiveness of the surface layers in SBF solution. As shown in Fig. 5b, high impedance barrier layers are evidenced with EIS. Tnts response is characterized by one time constant with

pseudocapacitive electronic behavior, with a slightly lower value of impedancecompared to T0. Heat treated TntsTT films present one time constant with higher phase angle and impedance modulus than Tnts, both indicating an increase of the barrier effect of the oxide, in accordance with anodic polarization results. The decrease in the phase angle at low frequencies for both anodized samples is consistent with a porous film as expected due to the open structure of the layers.

Since deterioration of the anodized surfaces could imply instability *in vivo*,electrochemical tests after 1, 14 and 28 days of immersion in SBF at 37°C according to ISO23317:2007(E)[24] were performed, and the results are presented in Fig. 6.

Anodic polarization curves of T0 present an increase in passivation potential after 14 days of immersion that remains after 28 days, with similar passive current density. EIS results of T0 show that the capacitive behavior of the native oxide remains and also does the protective effectiveness of the surface layer. These results indicate that the interaction between anions in SBF and native oxide do not deteriorate the barrier layer on titanium.Tnts stabilizes after 14 days of immersion with a low passive current that remains after 28 days. EIS results evidencethe evolution of the complex surface film structure during immersion time. These films do not behave as pseudo-capacitors, possibly due to the deterioration induced by the simultaneous growth/dissolution process during anodization. Remarkably, although TntsEIS shows changes in electrochemical response of the surface film during immersion time, that leads to changes in the bode plots, the current density remains almost unaltered. Finally, TntsTTsamples present low passivity current density, almost unaltered during immersion time and similar to those observed for Tnts after 14 days of immersion. Nevertheless, bode plots present important differences between TntsTT andTnts:while Tnts presents two defined timeconstants that characterizesa porous surface, the TntsTT suggesta closed structure evidenced by time constants very close or superimposed[42]. The annealed nanotube oxide behaves as a barrier layer with no major changes during immersion time. The stability of the films seems to increase due to thermal treatment, and this result, along with the increase in anatase leads a promising surface condition of TntsTT for further *in vivo* tests.

3.3.*In vivo* osteointegration of implants surface

3.3.1. Histological analysis and histomorphometry

The animals recovered perfectly well after the surgery and neither signs of infection nor inflammation were noted upon clinical examination during the experiment.

Fig. 7 shows optical microscopy images of Toluidine blue staining section of T0 andTntsTT implants30 days after surgery intibia and femur rat model.Four weeks after implantation, all implants are in direct contact with the surrounding bone, with no signs of inflammation at the bone implant interface.Neither mononuclear cell accumulations, nor osteoclast are seen close to the implants in both tibia and femur.No fibrous scar tissue is detected around the T0 and TntsTT implants. Inboth setsof samples, it is possible to distinguish the newly formed bone or the novo bone formation zone (with the implant in contact with the marrow medium). In this region,lamellar bone is in close contact with both implants (bone-implant contact). Although new bone formed around TntsTT implants showshomogeneoustissue all around the implants, there are areas where no bone was growth, displaying gaps between the new bone formations.

The histomorphometricquantitativeanalysis (Fig.8)reveals that after 30 days of implantation surgery, there is a slight decreasing tendency in the thickness of the new bone formed on TntsTT in both, tibia and femur bonescompared to T0. The results show no meaning dispersion in both bone condition analyzed.

3.3.2 Dynamic Histomorphometry

Sequential fluorescent labelling is usedto record and monitor new bone formation around the different implant groups by applying two types of fluorochromes. Fig.9 shows calcein and alizarin complexone deposition around the implants and it determined the mineral appositionprofile in tibia and femur rat model 30days after implantation. Fluorescent markers were detected in control T0 and annealed TntsTTimplants with the same irregular and intense distribution.Nonetheless, Fig. 10 shows the sequential fluorescent labeling where fluorochrome distribution exhibits an organized and clean profile in both conditionsfor tibia and femur bones.

Fig.10 shows MAR quantitativeresults 30days after surgery in the bone ingrowth around the T0 and TntsTT implantsboth in femur and tibia bones. The results show a tendency to a marked decrease in MAR in both bones on TntsTT when compared to the control.

4. Discussion

Among other properties, Titanium and its alloys are used for orthopaedic and dental applications as a consequenceof their ability to osseointegrateinto the surrounding bone[43,44].Latelyresearch has focusedthe attentionin surface engineered implants fabricated via anodization to generate self-ordered nanotubular structures composed of TiO_2[45].It has been demonstrated that the variations in the TiO_2 nanostructure could affect the adhesion, proliferation and cellular differentiation which was directly associated with the generation of bone tissue[46–48].Although those studies clearly support the modification of nanotubes as a suitable technique to improve the bioactivity of titanium implants, there is still some discrepancies over size and physico-chemical properties of TiO_2 nanotubes for optimal new bone formation and osseointegration.

From the crystalline and surface morphology analysis, our thermal treatment resulted successful in increasing the crystalline phases of Tnts with no substantial changes in nanotubes diameter, which remains between 82 and 89 nm. According to theRaman spectroscopy results, the increase in anatase after annealingmay be attributed to both the growth of anatase domains present in Tntsevidenced by the increase in Raman intensity peaks and also the appearance of new crystalline domains in TntsTT samples presenting anatase peaks over most of the analysed sites. Raman spectra also notices of some amount of rutileformed during heat treatment.

As important as biocompatibility, corrosion resistance is critical to determine the performance of a permanent implant. The presence of chloride ions in body fluids and their ability to induce localized corrosion on many metallic alloys, made human blood plasma a highly aggressive environment[49]. SBF was extensively used to evaluate the electrochemical behaviour of titanium *in vitro*, and its use in this work facilitates the comparison with literature results[50,51].There has been reported strong interactions between SBF or Hanks' solution ions and surface oxides on titanium[51,52], playing an important role in corrosion resistance after long period of immersion[53].

The film growth-dissolution process involved in the anodization of Tnts may alter the native film[54]and weaken the protection against corrosion in SBF solution, and be responsible for the

Tnts slightly higher current density and passivation potential than T0 that can be observed in figure 5.Moreover, the metal / oxide (M/O) surface alteration of Tnts surfaces is also evident when comparing the T0 and Tnts EIS results. The former, as expected, behaves as a non-ideal capacitive film, while the later present lower maximum phase angle and narrower frequency range with pseudocapacitive response.

Heat treatedTntsTT presents similar current density and passive behaviour as Tnts, but with a pseudocapacitive behaviour according to EIS. Since SBF contains several ions that may interact with the surfaces studied, EIS results not only describe the structure of the films but rather the film/ions interactionsare also represented and superimposed.Improvement in corrosion resistance in Ringer solution was observed after heat treatment by Saji et al.[55] and by Munirathinam et al.[56].

In T0, the native oxide formed in air is modified during immersion in SBF as evidenced in the increase in the passive potential. The effect of immersion looks similar to the obtained in aluminium during sealing immersion process[57]. No thickening of the oxide film results evident from EIS or polarization curves, since the growth of a protective layer often results in a diminish in current density and increase in impedance[58]. The initial condition (mechanical polish) lead to better results, regarding the corrosion resistance, than those obtained for *as received* titanium sheets[58]. EIS results after immersion present a slight decrease in phase angle in the low frequency region, often related to an increase in surface defects[59] accounting for the deleterious effect of SBF ions (chlorides mainly) on the surface oxide.

Our electrochemical results after 1 day of immersion are similar to those obtained by Hilario et al[60]. They performed apatite forming ability experiments as a bioactivity tests and found good results in anatase nanotubes. The excellent corrosion resistance of titanium was not dramatically changed by the nanotubular anodic film growth in this study. Nevertheless, T0, Tnts and TntsTT conditions presented high impedance and low corrosion density currents, both features of surfaces coated with a protective layer[61]. After 28 days of immersion, the three systems were stabilized with similar current densities and EIS results indicated that TntsTT behaves similarly to T0. Some deterioration of the anodic oxides is often observed

after immersion[62]. These results, presenting minor changes in electrochemical response and stability during immersion, are similar to those obtained by Krupa et al. on anodic films on titanium growth in electrolytes with Ca and P ions after 1000 hours of immersion in SBF[63].

If binding between an implant material and bone cannot be formed initially, then acceptance of the implant by the body, or more precisely the bone cell, fails. It is important to note that only 33–62% bone–implant contact is achieved by modern titanium implants with commercially available surface treatments after 3–6 months of implantation, indicating opportunities for improvements in osseointegration through research[64]. The focus of this study is to understand the influenceannealed TntsTTon the early osseointegrative properties of titanium implants in an animal model.It is widely known that the success of an implant strongly depends on the first cellular events, such as growth, proliferation and differentiation processes, which are regulated by specific genes and proteins associated with new bone tissue[65,66]. Thus, the success of osseointegration depends on the stable initial adhesion of osteoblasts onto the implant surfaces, which is a crucial prerequisite to subsequent cell functions such as synthesis of extracellular matrix proteins and formation of mineral deposits[67].

Our *in vivo* results indicated that both control T0 and TntsTT havelaminar bone growth on the surface of the implants in rat femur and tibia without significant difference in bone thickness 30 days after surgery. In accordance with these results, MAR showed no substantial changes between treatments inboth bones.Probably, changes in MAR and bone thickness could be noticed by the nanotubes presence only immediately after the implant is placed, since after 30 days post-surgery, only a plateau in bone formation is observed.Therefore, it is of mainimportance in further studies to evaluate the very initial cell adhesion properties on the nanotubes surface. In line with this, Li et al.[9] have evaluated the responses of rat mesenchymal stem cells to 75 nm diameter nanotubeswith different crystalline phases, including anatase, rutile, and anatase/rutile mixture. Theyshow that rat mesenchymal stem cells (rMSCs) had similar adhesion, proliferation, and differentiation behaviour on nanotubeswith different crystallinity. In addition, no noticeable differences in vinculin-associated focal adhesion points were observed between different samples. On the other hand, Roguska et al.[68] examined the adhesion of living cells (human osteosarcoma cell line—

U2OS) on TiO_2 nanotubes with different diameters and crystalline phases. Their results showed that cells adhesion was maximized when nanotube structure changes from pure anatase to an anatase-rutile mixture. Likewise, Ying LI., et al.[9] reported that the TiO_2 nanotube structure with 70nm diameter produced by anodization exhibits beneficial effects on osteoblast cell proliferation and differentiation and, in order to achieve better osseointegration, they combined hydroxyapatite to the TiO_2 nanotube structure. Their*in vivo* animal experiment in a rat model revealed that both nano-texturized surfaces were much compatible with the surrounding tissue than pure Ti surfaces. Additionally, Bjursten et al.[8] implanted discs with TiO_2 nanotubes and with Ti grit-blasted surfaces in compression on the flat cortical bone in the proximal anterior tibias of rabbits. They formed TiO_2 nanotubes withanatase,with 100 nm outer diameter by 80 nm inner diameter. After four weeks, TiO_2 nanotube implants showed significantly higher bone-implant contact area compared with the grit blasted implants and histological analysis confirmed new bone formation and increased levels of calcium and phosphorus on the surfaces of the nanotubes compared with the microtextured implants.

Conclusions

Commercially pure titanium was anodized in acidic media with fluoride leading to ordered nanotubular structures of titanium with tubes diameter of 89±24 nm (Tnts). Samples with heat treated (TntsTT) lead to an increase in anatase with small presence of rutile. Corrosion tests revealed that after 28 days of immersion in SBF there are no major alterations in the corrosion resistance of TntsTT when compared with the polished surface.

The *in vivo* results evidenced new osseous tissue in bothconditions implanted.In accordance with these results, mineral apposition rate (MAR) showed no meaning changes between treatments and in both bones. Probably, changes in MAR parameters and bone thickness would be affected by the nanotubes presence immediately after the implant is placed, reaching a plateau in bone formation 30 days post-surgery.

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Disclosures

The authors report no conflicts of interest.

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Table I. Surface treatments performed in this work and sample labeling.

Figure 1:SEM photomicrographs (Secondary Electron Imaging mode).a-c. Polished titanium (T0) appearance at different magnifications. d-f. Anodized oxide film as formed(Tnts). g-i. Anodized film after thermal treatment (TntsTT).Electron beam energy 3 kV.CTnts: nanotubes with close top, G-R: Regular machining grooves and ridges, OTnts: nanotubes with open top, T: tearing.

Figure 2: Algorithm for detection and measurement of size distribution of nanotubes diameters. a. Original image, b. Image where enhanced regions, c. Binary image, d. Minor and major diameter of the nanotubes, e. Histogram presenting size distribution of Tnts in micrograph a, f. g. Size distribution of inside nanotubes for a set of images with Tnts condition.

Figure 3: G-DRX spectra of – T0 – Tnts– TntsTT. Anatase peak position is pointed out.

Figure 4: Raman spectra. a. Tnts: intense anatase peaks. b. TntsTT: intense anatase as major

phase, with evidence of rutile presence.

Figure 5: a. Anodic polarization curves of – T0 – Tnts– TntsTTin SBF solution after 1 day of immersion. b. Bode plot corresponding to · T0 · Tnts· TntsTT in SBF solution after 1 day of immersion.

Figure 6: Anodic polarization curves and EIS bode plots of T0 (a,b), Tnts (c,d) and TntsTT(e,f) after 1, 14 and 28 days of immersion in SBF solution at 37°C.

Figure 7: Histology showing bone-implant interface in rat tibia and femur cross-section 30 days after the implantation. A-B: Tibia A: T0, B: TntsTT, and C-D: Femur. C: T0, D: TntsTT. (Staining: Toluidine blue).

Figure 8: Thickness of new bone layer on the implant surface in T0 and TntsTT 30 days after surgery.The data are shown in the form of mean value ± σ MEAN (standard deviation of the mean)n=6 bones per group (by non-parametric test. Man-Whitney). T0: control and TntsTT: annealed titanium oxide nanotubes.

Figure 9: Fluorescent microscopy image of rat tibia (A-F) and femur (G-L) cross-sections with T0 and TntsTT 30 days after surgery. Green (Calcein) and red (Alizarin Complexone) lines are seen in the new bone around the implant. BM: bone marrow, I: implant, T0: control; TntsTT:annealed titanium oxide nanotubes. Original magnification: 10x.

Figure 10: Quantitative analysis of mineral apposition rate (MAR) in T0 and TntsTT implants 30 days after surgery. The data are shown in the form of mean value ± σ MEAN (standard deviation of the mean) n=6 bones per group (by non-parametric test. Man-Whitney). T0: control and TntsTT: annealed titanium oxide nanotubes.

Table I. Surface treatments performed in this work and sample labeling

Sample denomination	Surface treatment		
	Polishing	Anodizing	Heat Treatment
Ti	Mechanical polishing with 400 and 600 grit paper, water as lubricant.		
Tnts	Mechanical polishing with 400 and 600 grit paper, water as lubricant.	Potentiostatic anodization in 1 mol.L^{-1} H_3PO_4 + 0.3 % HF solution, 30 V, 60 minutes.	
TntsHT	Mechanical polishing with 400 and 600 grit paper, water as lubricant.	Potentiostatic anodization in 1 mol.L^{-1} H_3PO_4 +0.3 % HF solution, 30 V, 60 minutes.	180 minutes at 350ºC in air atmosphere, and cooling in furnace.