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Biofunctionalization of Titanium to Improve the Response in Patients with Osteoporosis Requiring Implants

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Pathologic changes due to joint replacement in patients suffering from osteoporosis affect the bone microstructure and the implant stability. In order to assess changes in bone density as an improvement index of the trabecular microarchitecture, Ti6Al4V screws were biofunctionalized by electrospray (Electrostatic Spray Deposition) with TiO_2 nanoparticles (TiO_2np) or nanoparticles of TiO_2 and raloxifene (TiO_2np+R).
These treated screws were implanted into adult female Wistar rats at the proximal femur right and left, respectively. Untreated titanium screws were used in the distal portion of each femur as a negative control. Radiographic examinations of each femur were made 50 and 80 days post screw implantation and the regions of interest in the neighborhood of the bolts were extracted to compare the bone density of the distal (untreated) vs. proximal (treated) bolts and the proximal left (TiO_2np) vs. proximal right (TiO_2np+R) regions. Subsequently, the specimens were euthanized so as to correlate the images with both the microscopic and macroscopic changes from bone to implant.
The results of this preliminary study showed that the biofunctionalization with TiO_2np+R improves the response to the implant, increasing the osseous density from the radiological perspective and favoring the osseointegration from both a microscopic and macroscopic perspective.

1. Introduction

Osteoporosis is a disease of high socio-economic impact worldwide. When a joint replacement is performed a patient suffering from osteoporosis, pathologic changes in the microstructure of the trabecular bone affect the stability of the implant, making the negative impact this disease has on the response to the biomaterial evident.
The biofunctionalization techniques seek to modify the topographic and physicochemical characteristics of the surface of a material with the purpose of improving the biological response to the biomaterial (Arcos et al, 2014). The electrospray (Electrostatic Spray Deposition) is a technique that enables the biofunctionalization of materials through the atomization of a liquid by means of electric charges (Martin et al., 2012).
The material to be deposited is subjected to high voltages and can be atomized directly over the substrate by using (or not) a solvent, according to its characteristics (Clavijo-Grimaldo, 2012). Biofunctionalization of titanium surfaces with drugs is a strategy to decrease the incidence of complications in patients requiring orthopedic implants (Kyllönen et al,2015). Due to the fact that this is an area of recent research, no studies have been found where image processing is performed as an objective method to demonstrate the radiological changes of the peri-implant area, when titanium is biofunctionalized with raloxifene, medical product approved by the Food and Drug Administration (FDA) for the treatment of osteoporosis, to correlate them with micro and macroscopic changes. In this work we use the digital subtraction of radiographies (DSR) to compare the effects of the biofunctionalization with raloxifene in the mineral density of the bone near the implant (Carneiro et al., 2012).

2. Materials and methods.

2.1 Preparation of implants

Ti6Al4V screws with a length of 2.5 mm, 2.0 mm outer diameter and 1.5 mm inner diameter were cleaned in an ultrasonic bath in either acetone, ethanol and distilled water for 5, 10 and 10 minutes, respectively. The screws were biofunctionalized by electrospray using two solutions: one of nanoparticles of TiO_2 (TiO_2np) and another of TiO2 nanoparticles and raloxifene hydrochloride (TiO_2np+R). The TiO_2np, raloxifene hydrochloride and solvents were supplied by Sigma Aldrich. To achieve a homogeneous coating the concentration of the solution, voltage, flow rate and distance substrate were adjusted so that a stable cone configuration Tylor-Jet-Spray was obtained. The information on the solvents used in the solutions and the definitive deposition parameters have been protected by means of the patent application ((Universidad Nacional de Colombia-UNED, 2017). The immobilization of the TiO_2np and raloxifene was confirmed by Raman spectroscopy. Electron microscopy (SEM) was used to determine the morphological characteristics of the coating, obtaining images of the coating as well as its roughness (Average Roughness Rz y Arithmetic average Roughness Ra) and porosity using the software 3e-Reconstruction y Porometric, respectively.

2.2 Animal model

A total of 6 female Wistar rats, adult, average weight 230 g, were used for in vivo evaluation. The animals were anesthetized with isoflurane and screws were implanted into both femurs using a lateral approach. Where possible the screws were implanted in the same places, with reference to the muscle insertions on the femur but individual anatomical differences and the asymmetry due to right-left dominance make it very difficult for exactly the same location. Ti6Al4V screws biofunctionalized with TiO_2np and TiO_2np+R were implanted at the proximal femur right and left, respectively. Untreated titanium screws were used in the distal portion of each femur as a negative control. Specimen #0 was sacrificed at 24 hours, according to the FDA protocol in order to observe acute changes (FDA, 2017). Since no adverse effects at 24 hours were observed, the remaining five specimens remained with the implant until they were sacrificed at 80 days post-surgery. Radiographic examinations of each femur were made 50 and 80 days post screw implantation. Two animals presenting old bone fractures were excluded from this analysis. The study was performed in accordance with international standards (FDA, 2017) and with the approval of the Ethics Committee of Bioethics of the Faculty of Veterinary Medicine and Zootechnic of the Universidad Nacional de Colombia. For being considered a preliminary test, the number of specimens was determined by following the recommendations of the Three Rs Principle of the European Animal Research Association (EARA) (EARA, 2017).

2.3 Image acquisition and processing

Using a Siemens Polymat Plus 30/50 X-ray machine (52 kV, 10 mAs) and a digital radiography system (Agfa CR 30-X), radiographs of each femur at 50 and 80 days post implantation were taken, digitized and analysed. Before any comparison can be done, the portions of the image containing the rats' left and right femurs need to be aligned to a common geometrical reference. The metallic bolts were not used as a reference despite their high contrast and distinctiveness as the operation protocol does not guarantee the bolt position to be perfectly symmetrical or a constant orientation in the X-ray machine remain constant. Instead, an iterative retrospective registration of the femur was used. The proximal and distal areas of the femurs were segmented using the watershed lines 6 of the magnitude of the gradient image calculated using the Sobel operator. Two manually selected seeds where used for the distal and proximal femur areas (foreground) and a third one for the background area including the heads of the bolt (Figure 1). Images of the left femur were \mirrored" in the vertical plane to have the proper symmetry. An initial Euler rigid registration consisting of a composition of rotations and translations was used to align all left side image segmented femur masks (test images) to their respective right side masks (template images). The grayscale sum of squared differences (SSD) was used as the alignment criterion. The transformation resulting from the initial registration was used as a starting point for the affine registration of the original images. The segmented masks were used as ROI (Region Of Interest) and Mutual Information (MI) was used as the alignment criterion. Results were visually checked to insure a correct registration (Figure 2). Al registrations were done using the elastix software toolbox (Klein et al., 2010). Monofactorial variance analysis (ANOVA) was used to determine significant differences ($p<0.05$) between bone tissue response to biofunctionalized and untreated titanium screws by Analysis Lab software.

2.4 Evaluation of bone tissue response to implant

The euthanasia was carried out with a 100 mg/kg intraperitoneal dose of sodium pentobarbital. The femurs of the specimens were removed and immersed in 10% formalin. Subsequently, they were subjected to a decalcification process with Disodium ethylene diamine tetra acetic acid for two months. The screws were carefully removed and the complete femurs were included in paraffin. Longitudinal cuts of 5 microns thick were made and dyed with hematoxylin-eosin (H-E) to be observed under the light microscope.

3. Results

3.1 Characterization of the surface of implants

Figure 1 shows the morphology of the coatings obtained. A and D shows the surface of the TiO_2np coating (A) as seen through SEM (3000X) and the change in the morphology when the raloxifene is incorporated, TiO_2np+R (D). The surface becomes more uniform and the porosity is modified (B and E). With the Porometric software, it was determined that in the TiO_2np coating (B), the porosity is 35.26% with a pore diameter between 2.59 -13.1 µm and an average of 6.82 µm. The TiO2np+R coating (E), the porosity decreased to 22.5% with a pore diameter between 2.55-7.48 µm and an average of 3.12 µm. The high level of porosity and the surface area, incremented by the TiO_2 nanoparticles, were essential for the successful immobilization of the raloxifene. The 3d-Reconstruction software was used to evaluate the roughness (C and F), obtaining Rz values of 6.4-7.68 µm and Ra values of 2.35 to 3.21 µm for the TiO_2np coating (C) and Rz values of 11,28-13,5 µm and Ra values from 3.3-3.97 µm for the coating (F).

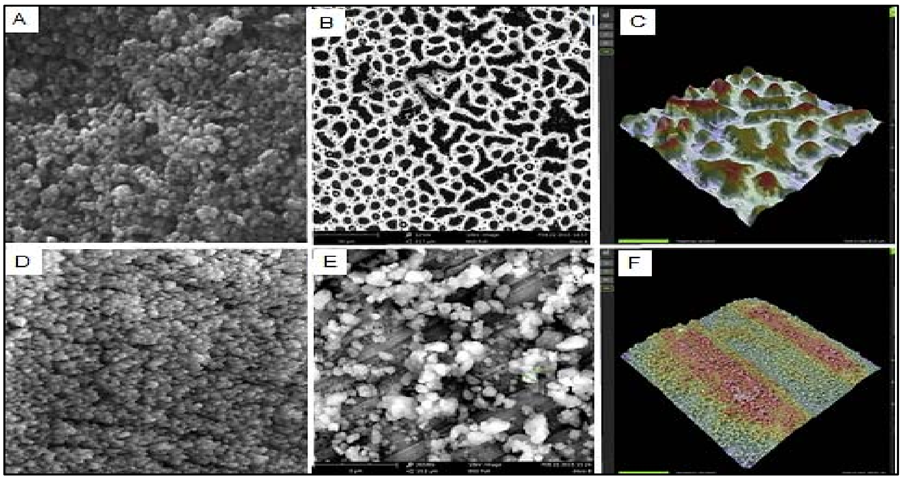

Figure 1: Morphology of coatings. Surface at SEM, 3000X (A), porosity (B) and roughness(C) de TiO_2np coating and surface at SEM, 3000X (D), porosity (E) and roughness (F) de TiO_2np+R coating

Figure 2 shows the Raman spectroscopy of the TiO_2np+R coating, obtained with an 875 nm wavelength laser. High intensities are observed at frequencies 639, 517, 397 and 145 cm^{-1}, which correspond to TiO_2. In the area between 1500 and 1650 cm^{-1} a deconvolution was performed to show frequencies 1612, 1594 and 1542 cm^{-1} which match the bands with the highest intensity in the raloxifene spectrum. In the frequency zone between 1040 and 1130 cm^{-1}, low-intensity peaks can be associated with the Ti-O-C functional group, where C belongs to raloxifene, which establishes this type of bond with the surface oxide.

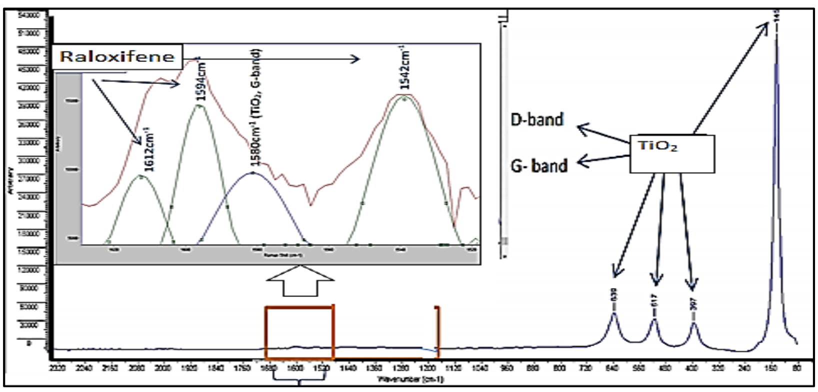

Figure 2: Raman spectroscopy of the TiO_2np+R coating

3.2 Image processing

Figure 3 shows contour of the segmented femur, overlapped registered images and to the Regions of Interest (ROI) were grayscale differences were measured. Although the femurs are correctly aligned, the bolts and surrounding bones are not.

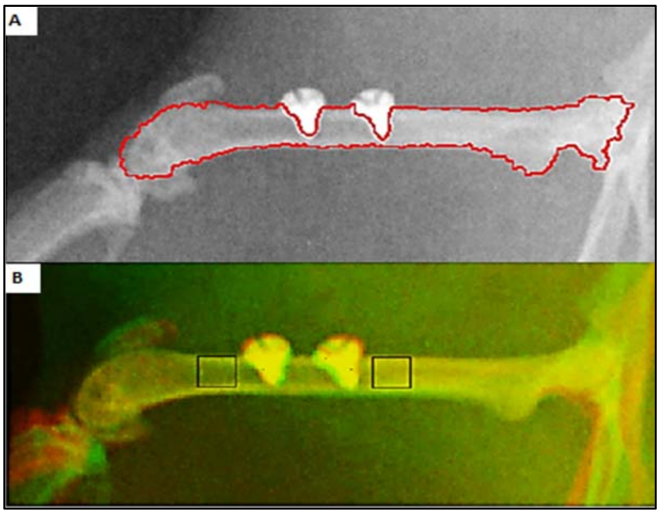

Figure 3. (A) Contour of the segmented femur. (B) Overlapped registered images: black boxes correspond to the Regions of Interest (ROI).

Table 1 shows the values for the proximal and distal ROIs in the right femur. When comparing the mean values of the grayscale within the ROI of the proximal ROI (biofunctionalized screw with TiO_2np) vs the distal ROI (untreated screw) at 50 and 80 days after the implant higher mean values are seen in the screws biofunctionalized with nanoparticles. However, the analysis of variance factor shows that the differences are not statistically significant: for the 50 days post-implant the p-value was p=0:057 and for the 80 days post-implant the p-value was p=0:051.

Table 1: Values for the ROIs in the right femur

Specimen	ROI_{p50}	ROI_{d50}	ROI_{p80}	ROI_{d80}
1	202.7	190.5	148.7	121.3
2	194.1	164.2	132.8	108.8
3	195.1	170.9	124.72	103.7

p: proximal, d: distal, 50:50 days post-implant, 80:80 days post-implant

Table 2 shows the values for the proximal and distal and proximal ROIs in the left femur. When comparing the mean values of the grayscale within the ROI of the proximal ROI (biofuncionalizad screw with TiO_2np+R) vs the distal ROI (untreated screw) at 50 and 80 days after the implant higher mean values are seen in the screws biofunctionalized. The analysis of variance factor shows that the differences are statistically significant: for the 50 days post-implant the p-value was p=0.0067 and for the 80 days post-implant the p-value was p=0.04. In this case the TiO_2np effect over the osteoblastic response is added to the anti-osteoclastic effect of raloxifen, blocking the lysis in the bone common to an inadequate osseointegration of the implant and, therefore, improving bone density.

Table 2: Values for the ROIs in the left femur

Specimen	ROI_{p50}	ROI_{d50}	ROI_{p80}	ROI_{d80}
1	196.8	156.2	139	96.,3
2	188	149.7	114.4	72.3
3	197.8	158	143.2	108.1

p:proximal, d: distal, 50:50 days post-implant, 80:80 days post-implant

3.3 Macro and microscopic morphology

Figure 4 shows the macroscopic appearance after the removal of the implants from the right and left femurs of a specimen #2 euthanized 80 days after the transplant. Neoformation of bone tissue is seen at the entrance of the orifice which is larger in the orifice of the biofunctionalized screw with the TiO2np+R coating. The microscopic appearance of the peri-implant bone tissue is also shown. Biofunctionalized implants with the TiO2np+R coating showed more organized trabeculae, with abundant osteocytes and osteoblasts organized on an active edge at the periphery of the neoformed bone and earlier osseointegration with the implant when compared to those coated only with TiO2np. In the presence of raloxifene there was also a active and functional remodeling and earlier peripheral angiogenesis.

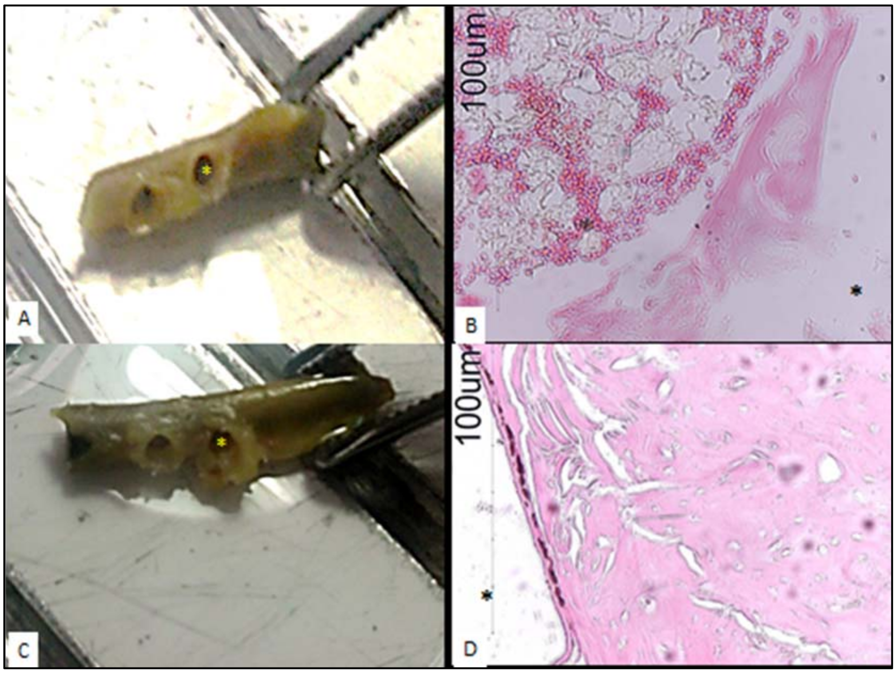

Figure 4. Macro and microscopic changes at 80 days post-implant. The asterisk indicates the site where the screw biofunctionalized with TiO2np (A-B) and with TiO2np+R (C-D).*

4. Conclusions

The results show a potential benefit of bone density around the site where the biofunctionalized screws were implanted. An increase in bone density, evaluated radiologically, is equivalent to an improvement in bone architecture, especially at the level of trabecular bone, which results in increased implant fixation. The results show that the screws biofunctionalized improve bone density in the peri-implant area when compared to non biofunctionalized screws. In average the incorporation of raloxifene had a greater effect on bone density (mean of the grayscale value within the ROI). Since titanium biofunctionalization using raloxifene is a recent research area (Harmankaya et al., 2013) there are no reports in the literature explaining variations in the response to such treatments or previous studies where image processing is used to analyze the results of the biofunctionalization implants with this drug to compare the effects in the mineral density of the bone near the implant. The results observed on bone density are due to the combination of the increase in the proliferation and viability of osteoblasts attributed to titanium dioxide and an anti-resorptive effect by modulating the osteoclast action own raloxifene.

The high incidence of osteoporosis worldwide and its impact on health and life quality shows the future studies are needed to establish the effects of biofunctionalization with drugs on bone quality and the possibility to reduce complications in patients with this condition (Vallet-Regí et al. 2017). The changes observed that allow the biofunctionalization of titanium with raloxifene is useful in the treatment of fractures in patients with osteoporosis due to its local effects in bone remodeling and in the osseointegration process. The biofunctionalization performed, although potentially useful to reduce complications and improve the quality of life of patients with osteoporosis undergoing joint replacements, is considered an initial phase and is necessary additional research.

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