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OSSEOINTEGRATION OF TITANIUM IMPLANTS ANODIZED WITH AND WITHOUT FLUORIDE IN THE ELECTROLYTE. A STUDY IN RATS

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Abstract

Based on the hypothesis that fluoride acts as a bone anabolic agent, the aim of this study was to measure in rats the osseointegration of implants (grade II titanium wire, 1 mm diameter, 4 mm long) submitted to anodic oxidation in 2 M phosphoric acid solution (*control implants*) or b) in 2 M phosphoric acid solution plus 0.2 M NaF (*F-modified implants*). Chemical composition of the implants surface was assessed by energy-dispersive X-ray spectroscopy. The surface of F-modified implants contained a 2.57% fluorine in weight. Adult male Sprague Dawley rats (300-350 g body weight) received two implants (in the femur and in the tibia, close to the knee) in each hind limb. Control and F-modified implants were inserted in the left and right hind limbs, respectively. Three weeks after surgery, the animals were sacrificed. The undecalcified bones were embedded in methylmetacrylate. Sections were obtained to measure two histomorphometric magnitudes: bone-to-implant contact (BIC) and bone volume in a defined volume of tissue around the implant (BV/TV). BIC was significantly increased on F-modified implants with respect to their controls (57.2%±3.3%, vs. 47.9±3.4, $p<0.05$). BV/TV did not differ significantly between F-modified and control implants (24.5±2.2% vs. 22.9±1.4, $p=0.30$). Profiles of the average gray pixel levels of pseudo3D images showed a greater roughness of F-modified implants respect to their controls ($p<0.05$). The relative contributions of surface roughness and its fluorine content to the osseointegration process requires further research.

Key words: implant, osseointegration, rat, fluoride.

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Resumen

OSTEO-INTEGRACIÓN DE IMPLANTES DE TITANIO ANODIZADO CON Y SIN AGREGADO DE FLUORURO EN EL ELECTROLITO. ESTUDIO EN LA RATA.

Con la hipótesis de que el ión fluoruro actúa como anabólico sobre las células óseas, el objetivo de este trabajo fue determinar el grado de osteo-integración (en la rata) de implantes (alambre de titanio II, 1 mm de diámetro, 4 mm de largo) anodizados en solución de ácido fosfórico 2 M + NaF 0,2 M (implantes-F) comparados con implantes controles, anodizados en solución de ácido fosfórico 2 M. La composición química de la superficie de los implantes fue evaluada mediante el espectro de dispersión de rayos X producidos durante la observación en el microscopio electrónico de barrido. La superficie de los implantes-F contiene 2.57% de flúor. Ratas macho Sprague-Dawley recibieron dos implantes (en el fémur y en tibia, próximos a la rodilla). Los implantes-F y controles se insertaron en las patas izquierda y derecha respectivamente. En los cortes de hueso sin decalcificación previa se midió el contacto hueso-implante (BIC) y volumen óseo en un volumen definido de tejido (BV/TV). BIC fue significativamente mayor con los Implantes-F respecto de los controles (57,2±3,3% vs. 47,9±3,4, $p<0,05$). BV/TV no exhibió diferencias significativas entre implantes-F y controles (24,5±2,2% vs. 22,9±1,4, $p=0,30$). Los perfiles de los niveles de grises de los imágenes pseudo3D de las superficies de los implantes pusieron en evidencia la mayor rugosidad de los implantes-F respecto de los controles ($p<0,05$). Las contribuciones relativas de la rugosidad y del flúor en el proceso de osteo-integración requieren investigación adicional.

Palabras clave: implantes, osteo-integración, rata, fluoruro.

Introduction

Ellingsen (1955)[1] first demonstrated that pretreatment with fluoride improves bone retention of implants. He "suggested that the presence of a fluoride coat on the surface of titanium implants stimulates the bone response leading to a connection between titanium and phosphate from tissue fluids. Free fluoride ions will catalyze this reaction and induce the formation of fluoridated hydroxyapatite and fluorapatite in the surrounding bone". Other reports (reviewed in the Discussion section) confirmed the positive effect of acid etching of the layer of titanium oxide with hydrofluoric acid or with alkaline fluoride + nitric or phosphoric acids solutions, producing implants with roughened surfaces, the common denominator of *second generation implants*. Anodizing of titanium is another method introduced in the second generation implants. This procedure increased the thickness of the titanium oxide layer, together with the production of a nanostructured surface with hydrophilic properties, rich in electrostatic charges given by the inclusion of phosphate ions in the titanium oxide layer.

Fluorosis showed that fluoride is a powerful bone anabolic agent. Two hypotheses (not mutually exclusive) have been published to explain the effect of fluoride on the proliferation of osteoblasts. Lau et al.[2] (1989) demonstrated that fluoride inhibits a protein-tyrosine acid phosphatase, responsible for the hydrolysis of phosphate in one or more signaling proteins of the MAP kinase cascade launched by action of hormones and cytokines. Other researchers[3-7] assigned activation of osteoblast proliferation to AlF_4, the complex of fluoride with aluminum, a trace element present in the circulation.

Many cell receptors have two associated G-proteins (stimulatory and inhibitory), which function is to control the initiation of the above-mentioned cascade. This control requires that the inhibitory G-protein have a GTP molecule in its structure. Aluminum fluoride replaces the γ-phosphate residue in guanosine diphosphate inhibitory G-protein and, as a consequence, the system remains stimulated. Present experiments were conducted to investigate whether fluoride present on the anodized surface of the implant stimulates osteogenic cells.

Materials and methods

Animals: Adult male Sprague Dawley rats, 300-350 g of body weight (11-week-old) were obtained from the vivarium of the School of Medical Sciences of the National University of Rosario. They were maintained in a controlled climate environment and fed with balanced chow and water *ad libitum*. All experiments were carried out in accordance with the guidelines on the NIH guide.[8]

Anesthesia, surgery and euthanasia

Pre-surgical preparation. Each rat received 0.25 ml of a 1 g/dl acepromazine solution per 100 g body weight by subcutaneous injection. Half an hour later the rat was placed in a 2.5 liters container together with a cotton swab embedded with 2 ml of isofluorane. Anesthesia took effect in 15 minutes.

Surgical procedure. Each rat received successive intramuscular injections: 0.3 ml of a ketamine solution (50 mg/ml), 0.1 ml of a diclofenac solution (25 mg/ml), and 0.1 ml of a ceftriaxone solution (30 mg/ml) per 100 g weight. During surgery, the snout of the rat was covered with a tube containing a swab with isofluorane.

The rat hind limbs were shaved and scrubbed with 10% povidone-iodine solution. The distal aspect of the femur and the proximal aspect of the tibia of each leg were carefully exposed via a skin incision and muscle dissection. Tissue was reflected to expose the flat portions of the femur and tibia, above and below the knee. The implant sites were prepared at 7 mm from the articular surfaces by hand drilling a hole, perpendicular to the bone surface, with a 1.1 mm diameter round bur. The implants were subsequently placed into the osteotomy and carefully pushed into place. After the correct implants positions were achieved, surgical sites were closed in layers. Muscle and skin were sutured separately with absorbable sutures. All rats recovered from surgery and displayed normal mobility and activity after 1 or 2 hours. Rats received standard rodent chow and water ad libitum. Analgesic was administered in the drinking water (0.25 g of diclofenac per liter) for one week.

Euthanasia. Each rat received, 0.25 ml per 100 g body weight of a acepromazine solution (1 g/dl), by subcutaneous injection. Half an hour later the rats were anesthetized as indicated above and then they were placed into carbon dioxide chamber for the time necessary for the death.

Implants: Titanium wire, grade II, 1 mm in diameter, was obtained at Roberto Cordes SA (Argentina). Raw wire lengths (40 cm) were submitted to two different anodizing conditions: a) in 2 M phosphoric acid solution (*control implants*) and b) in 2 M phosphoric acid solution plus 0.2 M NaF (*F-modified implants*).[9]

The wire (anode) was placed into a 500 ml plastic measuring cylinder internally lined with a 0.1 mm thick sheet of bronze (cathode). In order to ensure uniformity of the electric field, the titanium wire was centered into the cylinder with the aid of plastic discs attached at the ends. Anodizing was done at room temperature, constant 20 volts for one hour. After anodizing, implants were prepared by cutting the wire into 4 mm-long sections. They were cleaned by soaking in 96% ethanol for 24 hours and autoclaved.

Experimental design: Each rat received two implants in each hind limb. Control implants were inserted in the left leg and F-modified implants in the right leg. Three weeks after receiving the implants the animals were sacrificed to assess the osseointegration as described below.

Preparation of histological sections. After a healing period of 3 weeks, rats were euthanized as detailed above. The skin was incised on the medial side on the femoral-tibio-patellar region separating musculature from bone. Bone specimens (tibia and femur) were removed, cleaned of soft tissue, and fixed in phosphate-buffered paraformaldehyde solution for 12 hours. Subsequently, the specimens were dehydrated in an ascending series of ethanol (50–96%) over 2 days, cleared with xylene and finally embedded in methylmetacrylate, according to Maniatopoulus et al.[10] Once polymerized, blocks of acrylic were cut transversely to the implant axis, with a low speed metalographic saw (Isomet). The sections were made with a thickness of about 150 µm. Three to five cross sections were obtained of each implanted bone. The sections were thinned using 400 grit sandpaper and finally polished with 1000 grit sandpaper, lubricating with water. The 60 to 80 µm-sections were stained with of 2% Alizarin Red aqueous solution, for 5 minutes.

Histomorphometric analysis. Digital images of section were obtained using atrinocular light microscope (Leitz, Wetzlar, Germany). Digital images of sections were obtained at a 40x magnification with a camera (Olympus SP-350, China). Digital images were analyzed using the NIH image software.[11] Two histomorphometric parameters were determined in each section:

a) Bone-to-implant contact (BIC): BIC was measured around the implant (Figure 1A). Percent contact was defined as the length of bone contacting the implant, divided by the circumference length of the implant. Bone contact was defined as no visible gap at the light microscopic level. For this system, this represents any bone within 10 µm of the implant surface.

b) Bone volume within a defined volume of tissue around the implant (BV/TV). It is expressed as a fraction of the area occupied by bone within a ring (centered in the implant) 500 and 1000 µm of internal and external radios, respectively (Figure 1B).

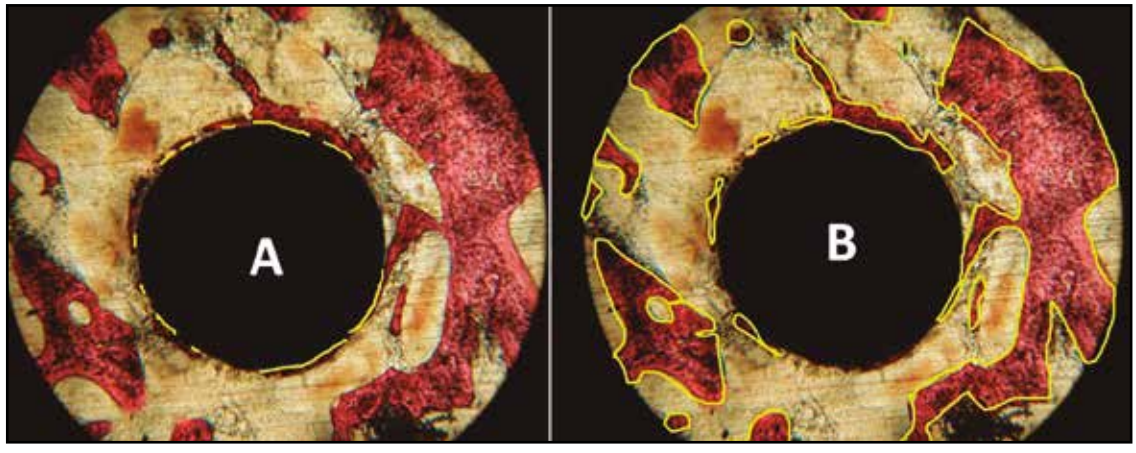

Figure 1. A: BIC. The lines around the circumference of the implant mark the lengths of bone-to implant-contact. **B:** BV/TV. The lines mark the areas of bone within the ring of standard dimensions around to the implant. The implant has a diameter of 1 mm.

Analysis of the implants surface at the Scanning Electron Microscope (SEM) and energy-dispersive X-ray spectroscopy (EDS).

Anodized (with or without fluoride in the electrolyte) and non-anodized titanium wire samples were examined by the scanning electron microscopy and the composition of the oxide layer was analyzed by EDS (spectroscopy energy dispersive).

Pseudo3D images of the surface of the implants. The RGB images of the implants (969x720 pixels) were converted to 16 bit-gray images. With the aid of the digital images analysis program, pseudo3D images of the surfaces of the implants were obtained as follows. The images were selected with the rectangle tool and a two-column table, summarizing the outline of the image was obtained. The column of the vertical axis (Y) contains the gray level average of the 720 pixels for each one of the 969 pixels of the X axis. Statistical analysis of the latter values gave the maximum and minimum values, the average and its standard deviation values and the 95% confidence interval of the average. These figures were used as surrogate variables to compare the surface roughness of the controls vs. F-modified implants.

Statistical analysis. Seven rats were used in these experiments, each one of which received four implants. Each implant produced 3 to 6 sections for histological analysis. Digital images of the sections were analyzed individually. The values of the measured variables were averaged for each implant. The percentage figures BIC and BV/TV were normalized by the angular transformation (angle = arcsine √percentage), before statistical analysis. The results were analyzed using the Student t-test.[12] Statistical significance was assigned if the value of $p<0.05$.

Results

Samples of raw and anodized titanium wire, with and without fluoride added to the electrolyte used in the process were observed under a scanning electron microscope. The Figure 2 reveals that anodizing modifies the roughness of titanium surface. The microanalysis of the elements present in the passivating layer (Table 1) reveals the presence of phosphorous and oxygen (from phosphoric acid) and fluorine in the F-modified wire, plus some contaminants granted, most probably, from the bronze anode.

Table 1. Elements composition at the surface of implants, assessed by energy dispersive spectroscopy.

Elements	Implants not anodized	Implants anodized in 2M H_3PO_4	Implants anodized in 2M H_3PO_4 + 0.2 M NaF
	Weight %	Weight %	Weight %
Carbon	12.81	23.31	18.00
Oxygen	n.d.	28.82	27.30
Fluorine	n.d.	n.d.	**2.57**
Sodium	n.d.	0.93	1.37
Magnesium	n.d.	0.49	0.80
Aluminum	1.91	1.69	1.77
Silica	n.d.	3.51	2.84
Phosphorous	n.d.	1.42	0.67
Titanium	85.27	38.59	44.46

n.d.= not-detected

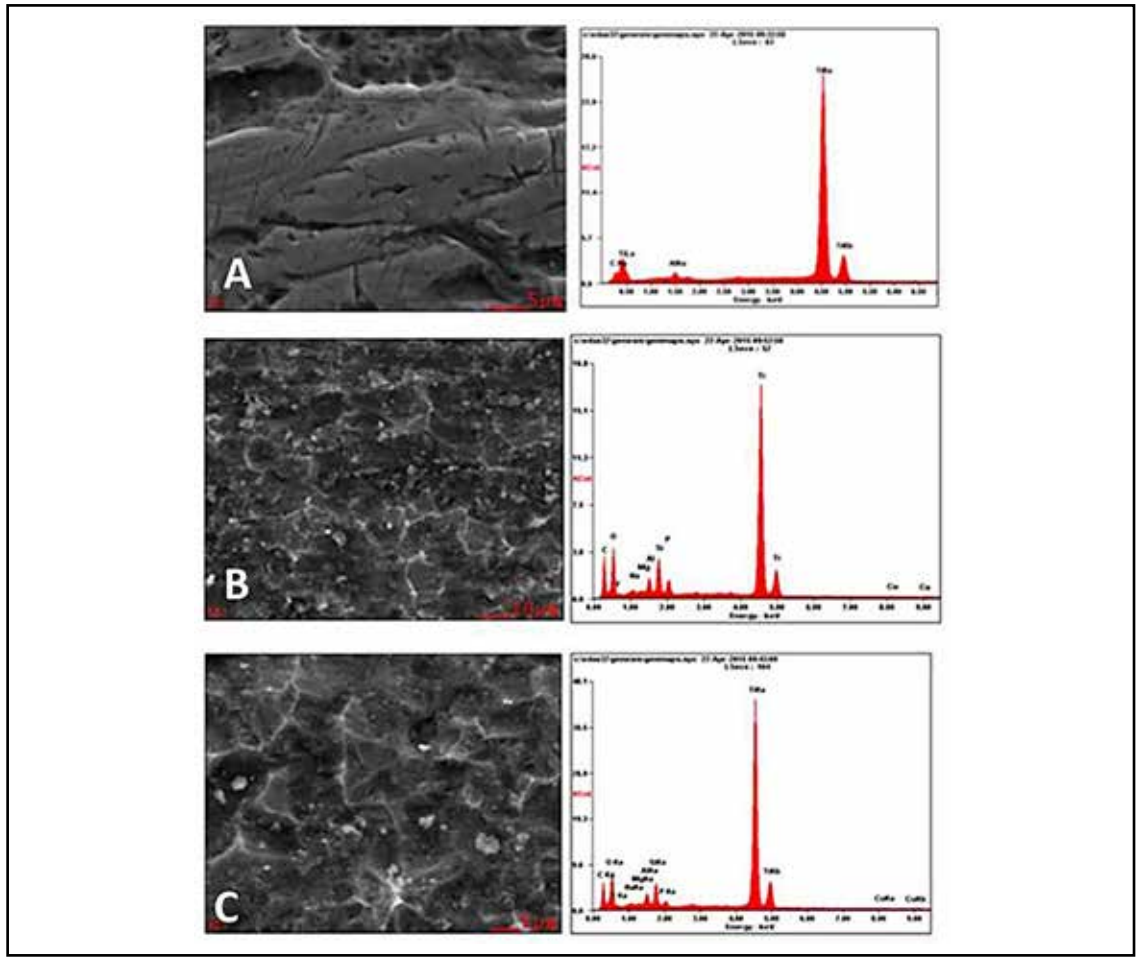

Figure 2. Left: A: surface of the coarse grained titanium wire. **B:** surface of anodized wire in phosphoric acid 2M. **C:** surface of anodized wire in phosphoric acid 2M+NaF 0.2M. **Right:** spectra of characteristic X-rays produced at the SEM.

The experimental model indicated that each rat received two implants in each hind limb. Control implants were inserted in the flat portions of the femur and tibia of the left leg, at 7 mm above and below the knee. F-modified implants were similarly inserted in the right leg. Three weeks after surgery the animals were sacrificed and bones were processed to assess osseointegration using two measures: BIC and BV/TV (Figure 1).

As expected, implants (control or F-modified) inserted in the femurs showed not significant differences with those of the tibia, either in the BIC or the BV/TV measurements (Tables 2 and 3).

When the pooled data of controls was compared with that of F-modified implants, significant differences were observed in the BIC variable and not in the BV/TV (Tables 4 and 5).

To compare the surface roughness of implants, the images of Figure 2 were converted to gray images of 16 bits to obtain the pseudo3D images. As described in Material and Methods a summary of the outline of the images of control and F-modified implants were obtained.

Table 2. Comparison of the osseointegration, assessed by BIC, of control (left femur and tibia) and F-modified implants (right femur and tibia) in the rat.

	Implants anodized in 2M H_3PO_4		Implants anodized in 2M H_3PO_4 + 0.2 M NaF	
	Tibia	**Fémur**	**Tibia**	**Fémur**
Number of rats	7		7	
BIC, mean±SEM, %	48.3±5.4	46.8±3.8	58.3±3.5	56.1±3.1
"t" test	0.611		1.245	
p value	0.554		0.237	

Table 3. Comparison of the osseointegration assessed by BIC, of control *vs.* F-modified implants, in the rat.

	Implants anodized in 2M H_3PO_4	Implants anodized in 2M H_3PO_4 + 0.2 M NaF
Number of implants	14	14
BIC, mean±SEM, %	47.9±3.4	57.2±3.3
"t" test	2.047	
p value	<0.05	

Tabla 4. Comparison of the osseointegration, assessed by BV/TV, of control (left femur and tibia) and F-modified implants (right femur and tibia) in the rat.

	Implants anodized in 2M H_3PO_4		Implants anodized in 2M H_3PO_4 + 0.2 M NaF	
	Tibia	**Fémur**	**Tibia**	**Fémur**
Number of rats	7		7	
BV/TV, mean±SEM, %	21.90±2.2	26.1±2.1	24.7±3.3	21.2±3.0
"t" test	1.381		0.750	
p value	0.159		0.434	

Table 5. Comparison of the osseointegration assessed by BV/TV, of control *vs.* F-modified implants, in the rat.

	Implants anodized in 2M H_3PO_4	Implants anodized in 2M H_3PO_4 + 0.2 M NaF
Number of implants	14	14
BV/TV, mean±SEM, %	24.5±2.2	22.9±1.4
"t" test	1.056	
p value	0.3007	

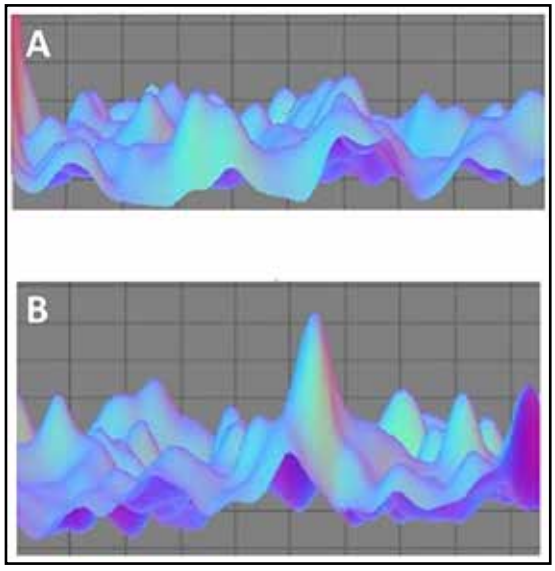

Figure 3. Pseudo3D images of the surfaces of anodized implant in phosphoric acid 2M (A) andin phosphoric acid 2M+NaF 0.2M.

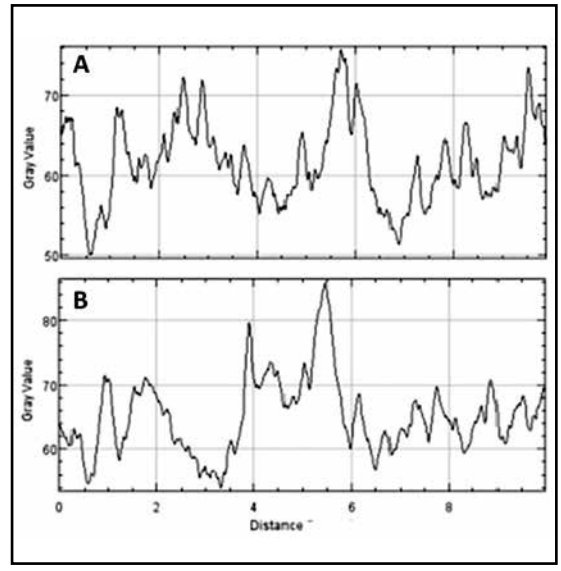

Figure 4. The graphs present the profiles of the average gray levels outline of the images of implants surfaces, previously converted to gray images of 16 bits. The ordinate shows the average gray level of the 720 pixels for each one of the 969 pixels of the width of the images. **A.** Implants anodized in phosphoric acid 2M; **B.** Anodized in phosphoric acid 2M+NaF 0.2M.

Table 6. Statistical summary of the profiles of average gray levels (surrogate variable of superficial roughness of implants) shown in Figure 4.

	Implants anodized in 2M H_3PO_4	**Implants anodized in 2M H_3PO_4 + 0.2 M NaF**
Minimum	3266	1429
Maximum	65470	65530
Mean ± SD	16350 ± 3484	16840 ± 3323
95% Confidence Interval of the mean	16100-16600	16590-17090

Statistical analysis of the outlines gave the maximum and minimum values, the average and its standard deviation values and the 95% confidence interval of the average. These data were used as surrogate variables to compare the surface roughness of the controls vs. F-modified implants. Inspection of Table 6 reveals that F-modified implants are significantly rougher than control ones ($p<0.05$).

Discussion

The concept of osseointegration was discovered by Brånemarket al.[15] and has had a great influence on the clinical treatment of oral implants. The first generation of titanium implants

had a machined surface. Shortly after, the second generation of implants appeared in the market. Clinical experience revealed that implants with a rough surface with homogeneous and uniform pores, gave the best molecular interactions, cell response and osseointegration.

The experiments reported in this paper investigate the effect of three particularities of the implants surface related to the osseointegration process: roughness, anodic oxidation and fluoride incorporation. A brief review of the literature on these particularities follows before reporting the results of present experiments.

Surface roughness. Surface roughness can be achieved with sand, Al_2O_3 or TiO_2 grit-blasting, coupled or not with acid etching, anodic oxidation and more recently with laser.[16-19] It has been proposed that the improvement in osteoconductivity of these strategies is related to the altered topography of the implant resulting in greater adhesion of osteoblasts and pre-osteoblasts.[20,21] The surface of implants should exhibit a microporous structure of about 0.5 to 1.0 μm diameter to facilitate insertion of osteoblasts filopodia. Additional micropores, 3 to 5 μm diameter allow osteoblasts to adhere strongly to those depressions. It is known, however, that the success of the implant depends on the complex environment that includes components of blood and other cells, not only osteogenic cells. So far, published clinical trials do not clearly describe whether the implants under investigation have machined or micro/nanotechnological surfaces.[14]

Anodic oxidation. The electrochemical process ofanodic oxidation provides two types of oxide layers as a function of the quality of the electrolyte employed to dissolve the oxide layer, A) nonporous films are produced with electrolytes in which the dissolution of the oxide is negligible and B) porous films are obtained with electrolytes containing acids in which the oxide is soluble. As the pores formed by anodic oxidation measure 10-100 nm, they are recognized as nanoporous structures.[20] The structural and chemical properties can be varied by controlling various parameters: anode potential, electrolyte composition, temperature, and current.[21] At lower voltage, a fairly constant growth of the oxide layer is obtained, while at higher voltage, gas evolution increases and thickening of the oxide layer is obtained.[22] Furthermore, depending on the electrolyte composition, different ions could be integrated into the oxide layer.[23,24] Anodic oxidation improves bone to implant contact and requires more torque to extract the threaded implants.[25,26]

Fluoride modified implants. In the 1995-2015 period, only one paper was published using fluoride modified implants in vivo. Ellingsen[1] reported that fluoridepre-treatment of titanium implants increased four times their retention in rabbits ulnas, after four and eight weeks of healing period, as measured by a push out technique. He F et al.[27] investigated the bone response to rough titanium implants treated with hydrofluoric acid/nitric acid (HF/HNO_3) solution. Two to 8 weeks after surgery, the tibias of rabbits were retrieved and prepared for removal torque testing and histomorphometric evaluation. In the same period, eight reports were published investigating the proliferation of pluripotent mesenchymal cells of different sources or the gene expression of osteoblasts *in vitro*.[28-33] Only two of these reports employed anodized titanium with fluoride modified surfaces. Jimbo et al.[33] reported the enhanced expression of genes involved in osseointegration in a culture of human osteoblast-like cell line. Kim et al.[32] investigating the behavior of pluripotent mesenchymal cells reported that surface roughness enhances the hydrophilic property of the anodized Ti and improves the initial cell response to it.

Present experiments. Anodic oxidation of the implants employed in this report were

performed in phosphoric acid solutions because it is less corrosive to titanium and it is associated with a most interesting feature: the reaction with and permanent presence of phosphate anions on the surface of titanium oxide. The 0.5 to 4 M phosphoric acid solutions contain un-dissociated acid and $H_2PO_4^{1-}$ ions exhibiting strong affinity to cations. Anodizing the implant in 2 M H_3PO_4+ 0.2 M NaF solution, as detailed by Krasicka-Cydzik et al.[9] modifies the surface of the implants: increases the thickness of the TiO_2 layer, incorporates hydrophilic quality and electrostatic charges to the surface providing a nanostructured platform for binding different proteins, modifies the topography and surface roughness, and incorporates fluoride to the oxide layer. The scheme of Figure 5 is based on the presumed reaction between phosphoric acid and titanium oxide. The chemical binding of fluoride in this structure, however, is as yet unknown.

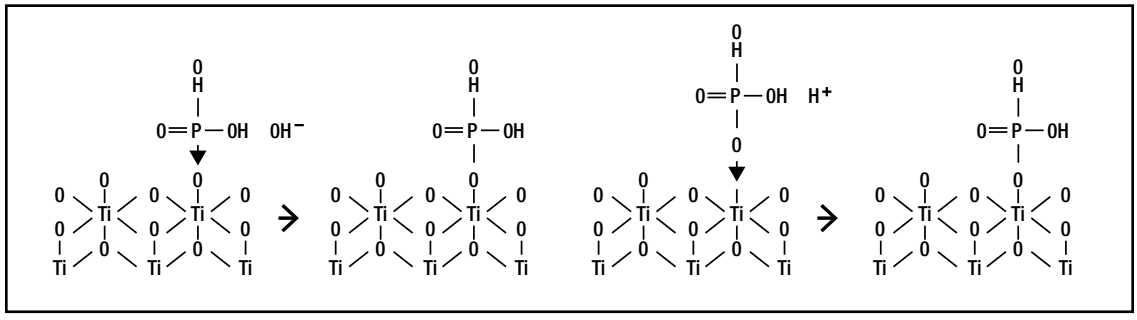

Figure 5. Theoretical scheme of phosphoric acid-titanium oxide structure, inferred from studies of electron spectroscopy.[13]

According to Puleo and Nanci,[14] bone formation occurs in the periprosthetic region in two directions simultaneously: from the implant to the bone (contact osteogenesis) and from the metaphyseal trabecular bone towards the implant (distant osteogenesis).

Contact osteogenesis was assessed by BIC (bone to implant contact). The results obtained indicate that F-modified surface significantly improves implant osseointegration, and agree with the report by Ellingsen et al.[1]

Anodizing with the incorporation of fluoride did not affect the BV/TV variable. It is not possible to draw a definitive conclusion on whether the inclusion of fluoride affected or not distant osteogenesis, a phenomenon that requires evaluation with the tetracycline labeling technique. According to Puleo and Nanci,[14] analysis of fluorochrome labeling demonstrates that the bone extending away from the implant forms at a rate about 30% faster than that moving toward the biomaterial.

The implants with F-modified surface differ from controls implants not only in their fluoride content but also in the roughness of their surfaces. These results raise the question on the fractional contributions of surface roughness and fluoride content on the proliferation of osteoblasts, as assessed by the BIC variable. Additional research is required to determine the relative contributions of the roughness of implant surface and its fluorine content to the osseointegration process.

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Conflict of interest: The authors have no conflict of interest to declare.

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References

1. Ellingsen JE. Pretreatment of titanium implants with fluoride improves their retention in bone. *J Mater Sci Mater Me*d 1995; 6:749-53**.**
2. Lau KH, Farley JR, Freeman TK, Baylink DJ. A proposed mechanism of the mitogenic action of fluoride on bone cells: inhibition of the activity of an osteoblastic acid phosphatase. *Metabolism* 1989; 38:858-68.
3. Caverzasio J, Palmer G, Bonjour JP. Fluoride: mode of action. *Bone* 1998; 22:585-9.
4. Jeschke M, Standke GJ, Scaronuscarona M. Fluoruroaluminate induces activation and association of Src and Pyk2 tyrosine kinases in osteoblastic MC3T3-E1 cells. *J Biol Chem* 1998; 273:11354-61.
5. Ammann P, Rizzoli R, Caverzasio J, Bonjour JP. Fluoride potentiates the osteogenic effects of IGF-I in aged ovariectomized rats. *Bone* 1998; 22:39-43.
6. Susa M. Heterotrimeric G proteins as fluoride targets in bone (review). *Int J Mol Med* 1999; 3:115-26.
7. Lau KH, Goodwin C, Arias M, Mohan S, Baylink DJ. Bone cell mitogenic action of fluoruro aluminate and aluminium fluoride but not that of sodium fluoride involves upregulation of the insulin-like growth factor system. *Bone* 2002; 30:705-11.
8. US Department of Health Services. NIH Publication No. 86-23 revised ed. Bethesda, MD: NIH 1985.
9. Krasicka-Cydzik E, Kowalski K, Kaczmarek A. Anodic and nanostructural layers on titanium and its alloys for medical applications. *Mater Eng* 2009; 5:132
10. ManiatopoulosC, RodriguezA, Deporter DA, Melcher AH. An improved method for preparing histological sections of metallic implants. *Int J Oral Maxifac Implants* 1986; 1:31-7.
11. Ferreira T, Rasband W. The imageJ user guide Version 1.43, Apr 2010. http://rsbweb.nih.gov/ij/docs/user-guide.pdf
12. Snedecor GW. Statistical methods. The Iowa State University Press, Ames, 1956.
13. Unal I. Phosphate adsorption on titanium oxide studied by some electron spectroscopy. Diploma,Universite de Geneva, Sept. 1999. Cited by Krasicka-Cydzik E., Kowalski K., Kaczmarek A. Anodic and nanostructural layers on titanium and its alloys for medical applications. *Mater. Eng*. 2009; 5:132.
14. Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. *Biomaterials* 1999; 20:2311-21.
15. Brånemark PI, Hansson BO, Adell R, Breine U, Lindstrom J, Hallen O, Ohman A. Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Scand J Plast Reconstr Surg* 1977; 16:1-132.
16. Cochran DL, Schenk RK, Lussi A, Higginbottom FL, Buser D. Bone response to unloaded and loaded titanium implants with a sandblasted and acid-etched surface: A histomorphometric study in the canine mandible. *J Biomed Mater Res* 1998; 40:1-11.

17. Jansen JA, Wolke JGC, Swann S, van der Waerden JPCM, de Groot K. Application of magnetron-sputtering for producing ceramic coatings on implant materials. *Clin Oral Implants Res* 1993; 4:28-34.
18. Palmquist A, Lindberg F, Emanuelsson L, Brånemark R, Engqvist H, Thomsen P. Biomechanical, histological, and ultrastructural analyses of laser micro- and nano-structured titanium alloy implants: A study in rabbit. *J Biomed Mater Res* 2010; 92:1476-86.
19. Brånemark R, Emanuelsson L, Palmquist A, Thomsen P. Bone response to laser induced micro- and nano-size titanium surface features. *Nanomedicine* 2011; 7:220-7.
20. Chehroudi B, Ratkay J, Brunette DM. The role of implant surface geometry on mineralization in vitro and in vivo: a transmission and electronmicroscopic study. *Cells Mater* 1992; 2:89-104.
21. Cooper LF, Masuda T, Yliheikkila PK, Felton DA. Generalizations regarding the process and phenomenon of osseointegration. Part 2: in vitro studies. *J Oral Maxillofac Implants* 1998; 13:163-74.
22. Andrade JD. Principles of protein adsorption. In: Andrade JD, editor. Surface and interfacial aspects of biomedical polymers. New York: Plenum Press, 1985. p. 1-80.
23. Lausmaa J. Mechanical, thermal, chemical and electrochemical surface treatment of titanium. 2001. In: Brunette DM, editor. Titanium in medicine: material science, surface science, engineering, biological responses and medical applications. Berlin: Springer, 1019.
24. Hall J, Lausmaa J. Properties of a new porous oxide surface on titanium implants. *Applied Osseointegration Research* 2000; 1:5-8.
25. Frojd V, Franke-Stenport V, Meirelles L, Wennerberg A. Increased bone contact to a calcium-incorporated oxidized commercially pure titanium implant: an in-vivo study in rabbits. *Int J Oral Maxillofac Surg* 2008; 37:561-6.
26. Sul YT, Johansson C, Byon E, Albrektsson T. The bone response of oxidized bioactive and non-bioactive titanium implants. *Biomaterials* 2005; 26:6720-30.
27. He F, Yang G, Zhao S, Cheng Z. Mechanical and histomorphometric evaluations of rough titanium implants treated with hydrofluoric acid/nitric acid solution in rabbit tibia.*Int J Oral Maxillofac Implants* 2011; 26:115-22.
28. Bibby JK, Bubb NL, Wood DJ, Mummery PM.Fluorapatite-mullite glass sputter coated Ti6Al4V for biomedical applications.*J Mater Sci Mater Med* 2005; 16:379-85.
29. Cooper LF, Zhou Y, Takebe J, Guo J, Abron A, Holmen A, Ellingsen JE. Fluoride modification effects on osteoblast behavior and bone formation at TiO2 grit-blasted c.p. titanium endosseous implants. *Biomaterials* 2006; 27:926-36.
30. Isa ZM, Schneider GB, Zaharias R, Seabold D, Stanford CM. Effects of fluoride-modified titanium surfaces on osteoblast proliferation and gene expression. *Int J Oral Maxillofac Implants.* 2006; 21:203-11.
31. Guo J, Padilla RJ, Ambrose W, De Kok IJ, Cooper LF. The effect of hydrofluoric acid treatment of TiO2 grit blasted titanium implants on adherent osteoblast gene expression in vitro and in vivo. *Biomaterials* 2007; 28:5418-25.
32. Kim CS, Sohn SH, Jeon SK, Kim KN, Ryu JJ, Kim MK. Effect of various implant coatings on biological responses in MG63 using cDNA microarray. *J Oral Rehabil* 2006; 33:368-79.
33. Jimbo R, Sawase T, Baba K, Kurogi T, Shibata Y, Atsuta M. Enhanced initial cell responses to chemically modified anodized titanium. *Clin Implant Dent Relat* Res 2008; 10:55-61.