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Bone regeneration around implants with modified surface by acid conditioning with the fluoride ions deposition

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ABSTRACT

Aim Evaluate the late bone regeneration around implants with machined (MS) and acid modified surfaces followed by the deposition of fluoride ions (AFS) in the tibiae of rabbits.
Materials and methods Ti-cp disks and implants underwent the topographic characterization before surgery through scanning electron microscope, x-ray energy dispersive spectrometer (SEM-EDX), roughness average (Ra) and, cross section. Six rabbits received 12 implants in their tibiae. After 12 weeks, euthanasia was performed. The percentage of bone interface contact (BIC%) and neoformed bone area (NBA%) was measured. Data were submitted to the statistical analysis.
Results SEM-EDX revealed smooth surface, contaminated with machining residues and peaks of Ti for MS group and AFS group, peaks and valleys with presence of Ti, O, Na, Cl and F. Ra showed statistical difference between AFS (1.34 ± 0.35 µm) than MS (0.4 ± 0.06 µm). Cross section showed a mean thickness of 7.84 ± 3.73 µm for ASF and 1.26 ± 0.55 µm for MS. The mean values of BIC% for AFS were statistically higher than MS. For NBA% there is no statistical difference between grups.
Conclusions The surface modified by acid conditioning followed the addition of fluoride ions provides superior osseointegration process, even in the later periods of bone regeneration.

KEYWORDS Bone-implant interface; Bone regeneration; Dental implants; Fluorine; Titanium.

INTRODUCTION

Dental implants have presented several changes as in their constitution, shape, geometry and surface modifications. Due to the intimate contact with biological tissues at the implantation site, the material surface is a critical factor for determination of biocompatibility and osseointegration process (1). Biological events at the bone-implant interface are influenced by several implant characteristics, such as chemical composition, surface energy and topography (2). Different topographies are produced by additive and subtractive processes and can result in the formation of reliefs at the micro, submicron and nanometric scales (3,4).
Immediately after implant installation, the interaction between the cells and the implant surface defines the cell-implant interface and may eventually affect the final bone-implant interface (5). The initial repair phase and subsequent osseointegration depends on the availability of the osteogenic cells and their adhesion capacity, as well as proliferation on the surface of the implant (6). Modifications in surface topography are directly related to cellular interaction, favoring cell adhesion, proliferation and differentiation. The most used modifications are represented by sandblasting, acid conditioning, anodizing, calcium phosphate coating and plasma spray (7-9), among others.
Microtopography helps bone formation because it generates a favorable three-dimensional environment for interactions between cells and extracellular matrix (10). Studies with osteogenic cell culture cultured on the titanium surface showed that the gradual increase of microtopography provides reduction of proliferation and increased cell differentiation, resulting in high levels of alkaline phosphatase (ALP) activity and osteocalcin synthesis (2). There is also an increase in the production of growth factors that promote osteogenesis, such as TGF-β1 (3), and molecules that reduce the formation and activity of osteoclasts, such as osteoprotegerin

(OPG), secreted by osteoblasts (11). In addition, titanium surfaces with microtopography, obtained by acid etching activate platelets and retain the fibrin clot more efficiently when compared to machined surfaces, which is important in the early stage of integration of materials with biological tissues *in vivo* (12).
The incorporation of ions on the surface of the implants aims to optimize the adhesion of osteoblasts and to produce an antibacterial surface by the anodic deposition of these ions (13), besides the production of more reactive surface layers (14). Treatment of the implant surface with fluoride is a method capable of increasing biocompatibility (4) and the incorporation of this ion can be accomplished by means of electrolytic treatment, immersion in solution containing fluoride or by means of the acid attack. Thus, the aim of this study was to evaluate the late biological behavior of bone tissue around implants before machined surfaces and modified by acid conditioning followed by deposition of fluoride ions installed in rabbit's femur.

MATERIALS AND METHODS

This study was submitted to and approved by the Ethics Committee on Animal Experimentation of the School of Dentistry, Sao Paulo State University, Araçatuba, Brazil, under the Process FOA-00824-2018, and was designed in accordance with the ARRIVE guidelines (15).

Animal model

In this experimental study, six white male rabbits (Oryctolaguscuniculus, New Zealand), albinus, aged approximately 5 months and body weight between 2.5 and 4 kg were used. The animals were kept in individual cages with standard diet, solid feed (Procoelho, Primor) and water "ad libitum" at the animal facility of the São Paulo State University, School of Dentistry, Araçatuba (FOA-UNESP). Power of the sample was considered at a significance level of 5% (with standard deviation of 2%), and with a test power of 80%. Alpha was defined as 0.05 and 6 rabbits per group were required for the purpose of comparing different groups.

Implants

Twelve cylindrical implants were used, with triangular threads and internal hexagon connection of 1.17 mm, with dimensions of 2 mm in diameter and 5 mm in length (Connection of Prosthesis Systems, São Paulo, Brazil), with two different surfaces.

- Machined Surface (MS): Commercially pure titanium implant Ti-cp and machined surface (Conexão Sistemas de Prótese, São Paulo, Brazil).
- Acid Fluoride Surface (AFS): Implanted Ti-cp modified by acidic conditioning followed by the deposition of fluorine ions (Porous-nano, Conexão Sistemas de Prótese, São Paulo, Brazil).

Implants of commercially pure titanium (Ti-cp) were immersed sequentially in acid baths (sulfuric acid – H_2SO_4, hydrochloric acid – HCl, and nitric acid –HNO_3) with previously established increasing concentrations constituting industry secrecy. After modification by acid conditioning the implants were immersed in NaF (sodium fluoride) solution in purified water. After a period of 60 minutes, the implants were withdrawn from the solution and rinsed in purified water, followed by bathing in ethyl alcohol. The implants were placed in an oven at 80 ° C for two hours for the drying process. Finally, the implants were sterilized by gamma rays and packed.

Surface topography characterization

The surface topography of the implants was analyzed before the installation in the femur of the rabbits by scanning eletron microscope (SEM, XL 30 TMP model, FEG, Philips XL Series, with detector Oxford incaX-sight, Netherlands, 97), coupled to x-ray energy dispersive spectrometer (EDX), for semi-quantitative analysis of the chemical composition of surfaces.
Ti-cp discs were modified with the same experimental surface. The average roughness (Ra) were analyzed on three discs of each group through a digital rugosimeter (Mitutoyo SJ-400, MitutoyoSul Americana Ltda, São Paulo, Brazil). Ten measurements were performed on each disc. In addition, a cross-sectional analysis was performed by SEM to determine the average thickness of 10 points measured on the surfaces of sectioned discs of each group.

Surgical procedure

Before the procedure the animals fasted for 8 hours prior to anesthesia. The animals were anesthetized by intramuscular infiltration (IM) with 50mg/kg of ketamine hydrochloride (Vetaset – Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil) and 5mg/Kg of Xylazine Hydrochloride (Dopaser – Laboratório Calier do Brazil Ltda – Osasco, São Paulo, Brazil). Then, the trichotomy was performed on both, rigth and left leg followed by antisepsis with polyvinyl pyrrolidone iodine degermant (PVPI 10% Degermante, Riodeine, Rioquímica, São José do Rio Preto, Brazil) and PVPI topic (PVPI 10% Tópico, Riodeine, Rioquímica, São José do Rio Preto, Brazil). Subsequently, the animals received local anesthesia through infiltration of mepivacaine hydrochloride (0.3 ml/Kg, Scandicaine 2% with adrenaline 1:100.000, Septodont, France) to aid in hemostasis.
A linear incision (2 cm) was performed parallel to the long axis of femur, bilaterally, 2 cm above the tibiofemoral joint in the dermis. Then, the divulsion of the muscle tissue was performed until the periosteum was exposed, which was then incised to expose the bone tissue The perforations were prepared by means of a reduction angle 20:1 (Kavo Brazil, Florianópolis, Brazil) coupled to an electric motor (Conexão Sistema de Próteses, São Paulo, Brazil) at 1200 rpm. The progressive sequence of milling, lance, helicoidal of 1.8 mm was carried out with abundant irrigation of 0.9% sodium chloride solution (Darrow, Rio de Janeiro, Brazil).

Then, an implant of each surface type was implanted per femur at a speed of 20 rpm. Suture was performed in in 2 layers, using absorbable yarn (Poligalactina 910 – Vycril 4.0, Ethicon, Johnson Prod, São José dos Campos, Brazil) with continuous stitches in the muscular layer, and non-absorbable yarn (Mononylon 4.0, Ethicon, Johnson, São José dos Campos, Brazil) with interrupted stitches on the dermal layer.

In the postoperative period, the animals received of 10 mg/kg IM of Enrofloxacin (Baytril, Bayer AS, São Paulo, Brazil), 0.2 mg/kg of meloxicam (Maxicam, Eurofarma Laboratórios AS, Ribeirão Preto, São Paulo, Brazil) e 0.1 mg/kg of butorphanolol (Torbugesic, Fort Dodge Saúde Animal Ltda, São Paulo, Brazil) in a single dose. The animals were evaluated 5 days a week for observation of clinical signs during the 12 weeks of the experiment. After 12 weeks after implant installation, the animals were euthanized by intraperitoneal administration of 150 mg/kg of pentabiotic sodium (Pentabiótico Veterinário, Fort Dodge Saúde Animal Ltda, São Paulo, Brazil).

Histometric analysis

After euthanasia, the tissue samples from the femurs containing the implant were removed. The pieces were fixed in neutral 10% buffered formalin (Reagentes Analíticos®, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) and underwent decalcification in EDTA 20% (Ethylenediaminetetraacetic acid; Merck, Darmstadt, Germany) dissolved in MiliQ water, with weekly exchanges, at room temperature. The samples were then dehydrated using a gradual and increasing alcohol concentration sequence (70, 90, 95 and 100%), with solution exchange every 1 hour on an orbital shaker (KLine CT – 150®, Cientec – Equipamentos para Laboratório, Piracicaba, SP, Brazil). Subsequently, the diaphanization with xylol and posterior, inclusion in paraffin to obtain slices of 6 µm thickness mounted on glass slides for the staining in hematoxilin and eosin (HE Merck & Co., Inc., NJ, EUA).

The slides were analyzed under an optical microscope (DIASTAR, Leica Reichert & Jung products, Germany) paired with an image capture camera (Leica Microsystems DFC-300-FX, Germany), with resolution of 1.3 megapixels, attached to the common light microscope and to the computer. The histometric analysis was performed through the Image J® (U.S. National Institutes of Health, Bethesda, MD, USA). The area of newly formed bone (NBA%) and the bone interface contact (BIC%) was calculated in percentage throughout the implant, located in cortical bone.

Statistical analysis

The values obtained from average roughness (Ra), NBA% and BIC% were tested by the Kolmogorov-Smirnov for evaluation of homostatistic, and afterwards, were compared using Tukey's multiple comparison test ($p<0,05$).

RESULTS

Topography of implant surfaces - SEM and EDX

Scanning electron microscopy showed that there was a topographic difference between the surfaces. MS presented a smooth surface topography, contaminated with machining debris (Fig. 1), while AFS presented a

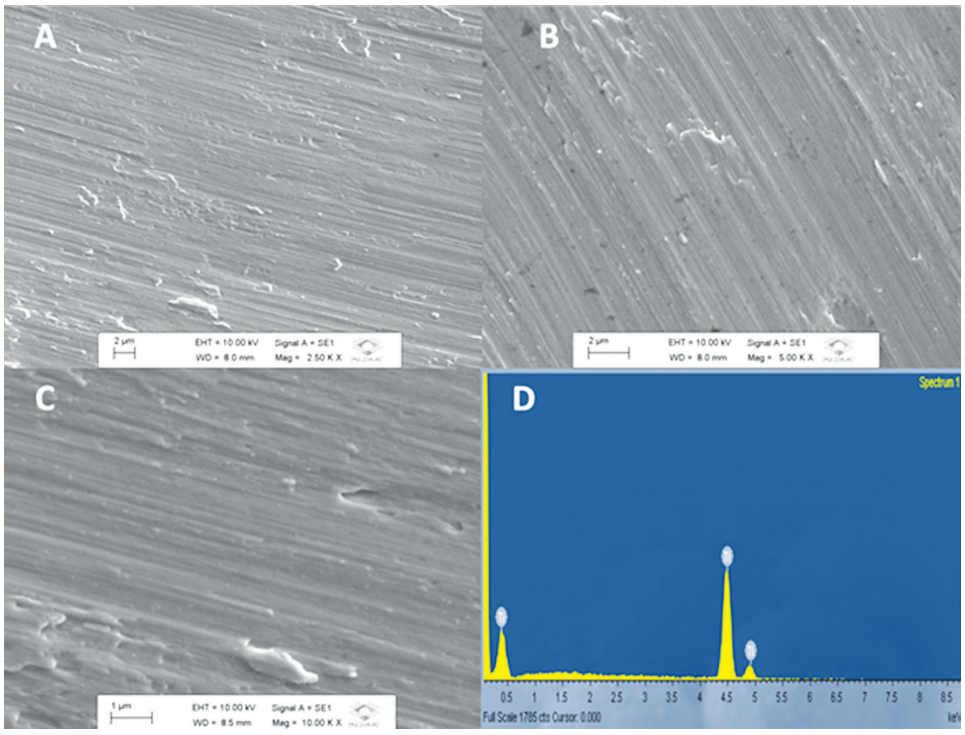


FIG. 1 SEM images at 250x (A), 500x (B) and 1000x (C) and Ti peaks evidenced by the the x-ray energy dispersive spectrometer (EDX) (D) for the MS experimental group.

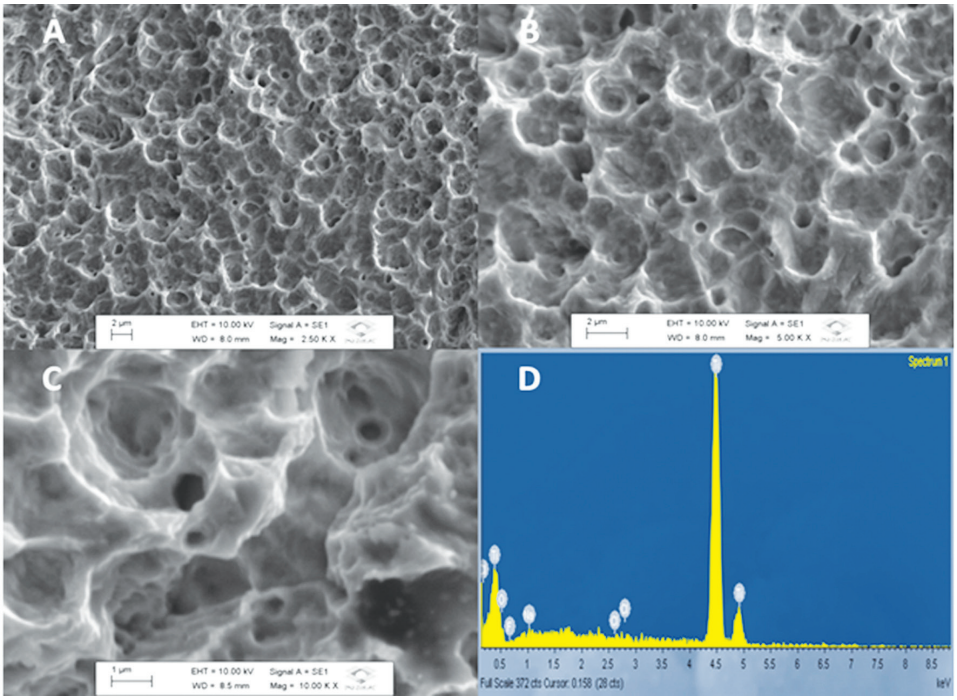
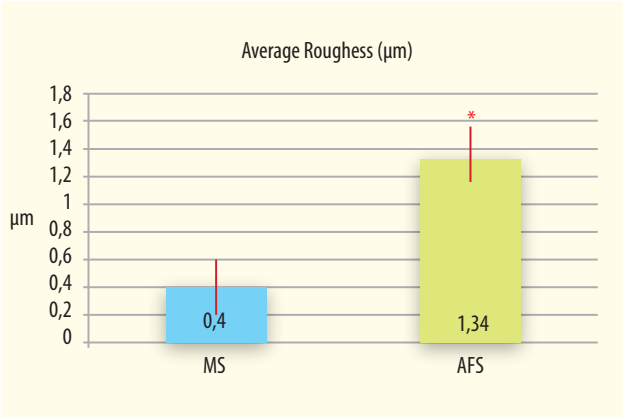


FIG. 2 SEM images at 250x (A), 500x (B) and 1000x (C) and Ti peaks evidenced by the x-ray energy dispersive spectrometer (EDX) (D) for experimental group AFS.

topography with a morphological pattern more rugged with presence of micro cavities surrounded by sharp micro peaks, similar to peaks and valleys (Fig. 2).
EDX analysis for MS surface showed no contamination and Ti peaks (Fig. 1). For AFS, they showed higher peaks of Ti and O and smaller peaks of Cl, Na and F (Fig. 2). It was not possible to visualize in the SEM the fluoride scavenging layer even in larger magnifications. However, it was possible to visualize the element in EDX analysis.

Roughness and roughness in cross section

The microtopographic analysis revealed a statistically significant difference ($p<0.05$) between roughness of AFS (Ra=1.34 ± 0.35µm) and MS (0,4 ± 0,06 µm) (Fig. 3). The cross section of the disks showed a statistically significant difference ($p<0.05$) between average thickness of 7.84 ± 3.73 µm for ASF, and 1.26 ± 0.55 µm for MS (Fig. 4).



** indicates a statistically significant difference (p <0.05)*

FIG. 3 Graph of the mean roughness values (Ra) and standard deviation of the MS and AFS groups.

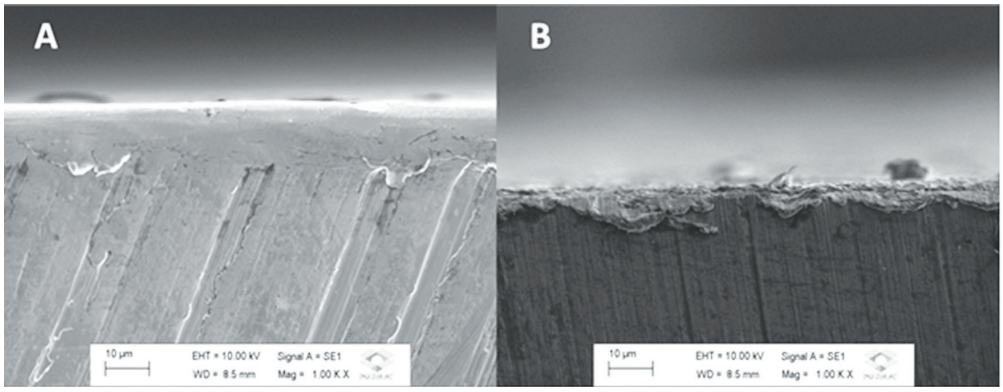


FIG. 4 SEM cross section of the disks of the MS (A) and AFS (B) groups (1000x).

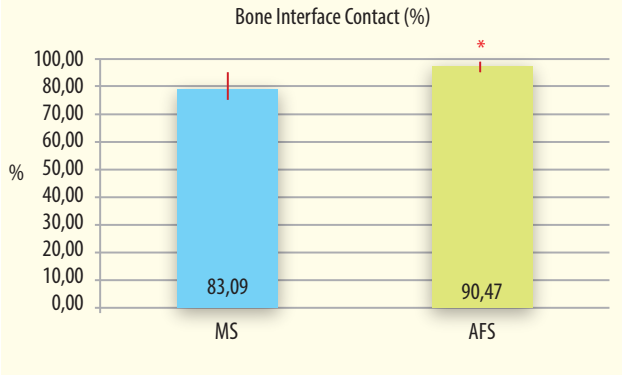


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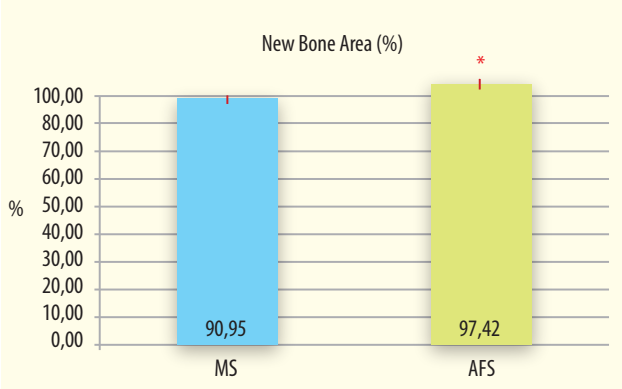
FIG. 5 BIC% graph and standard deviation for the MS and AFS groups.



** indicates a statistically significant difference (p <0.05)*

FIG. 6 NBA% graph and standard deviation for MS and ASF groups.

Histometric analysis

The average values in percentage of bone interface contact (BIC%) for MS surface were 83.09%, while for AFS the average values were 90.47% (Fig. 5). The mean BIC% values for the AFS group were statistically higher ($p = 0.0443$) when compared to the mean values of the MS surface. The mean values in percentage of new bone area (NBA%) for MS surface were 90.95%, while for AFS the average values were 97.42% (Fig. 6). The NBA% values were not statistically significant between MS and AFS ($p = 0.0004$).

In the AFS group concentric lamellae were observed suggesting an advanced process of bone mineralization, presence of greater bone mineralization at the interface, suggesting lamellar compaction. In the MS group, a greater amount of connective tissue was observed at the interface between bone tissue and implant, suggesting that this tissue is at a less advanced stage when compared to AFS. In some points concentric lamellae were observed, however in a smaller number when compared to the AFS group (Fig. 7).

DISCUSSION

The modifications in the titanium surface of the dental implants aim at an optimization of the osseointegration in order to generate an advance of the prosthetic phase or to enable rehabilitation by the immediate loading technique. De Franco et al. (2012) (9) demonstrated that the implant surface modification treated with organic acids can represent a good solution for the prosthetic rehabilitation of partially and completely edentulous

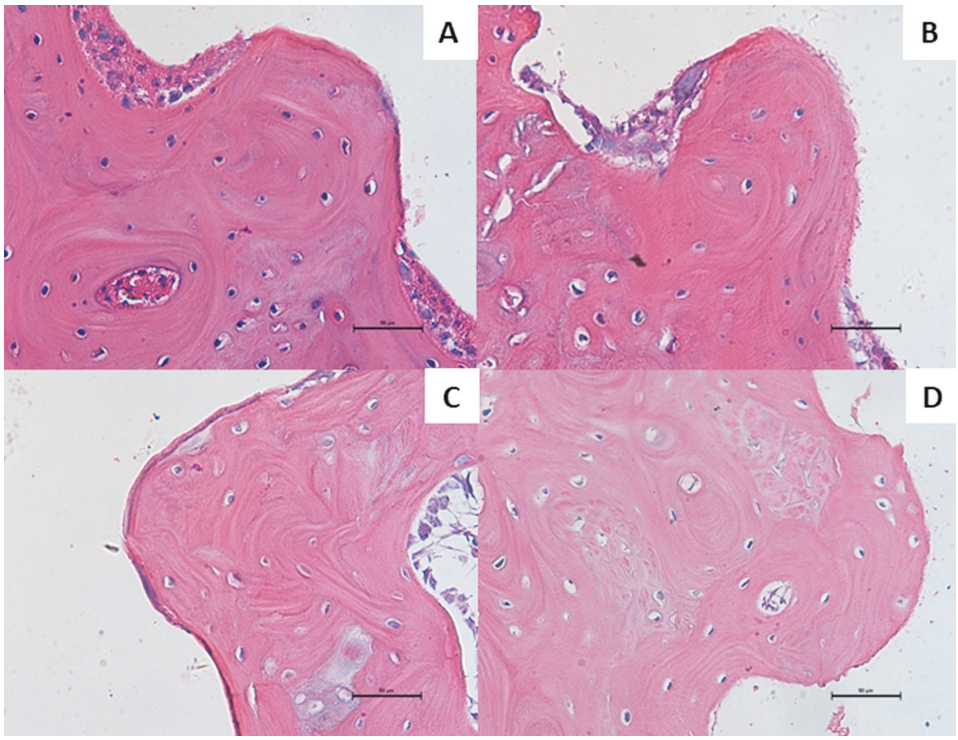


FIG. 7 Decalcified histological sections of the delta region of the implants of the MS (A and B) and AFS (C and D) implants located in the cortex of the tibiae (hematoxylin and eosin, 40x).

patients with a success rate of 96.07%.
Thus, it is possible to emphasize the surface modification by acid conditioning followed by the addition of fluorine ions (4). This type of surface modification provides a more reactive surface layer, superiority of biocompatibility, modulation of bone neoformation, stimulation of osteoblast proliferation and increase of the affinity of the titanium dioxide layer to the calcium and phosphate ions, which consequently favor the deposition process and bone formation (12,16,17), as observed in this experimental study in which the bone implant contact in percentage (BIC%) and neoformed bone area (NBA%) values of the AFS group were statistically superior ($p < 0.05$) when compared to the MS group.
In a previously published study (18) on peri-implant defects with a 50% extension of the buccal wall, followed by the installation of implants modified by blasting with a titanium oxide particle or implants modified by the addition of fluoride, after 6 weeks, the histomorphometric analysis revealed higher bone implant contact in percentage (BIC%) and neoformed bone area (NBA%) in the fluoride addition implants. Similarly, the AFS experimental group of this study presented values statistically superior to the MS experimental group ($p < 0.05$).
A review of the literature (19) pointed out factors correlated to the surface roughness of the implant and the biological response, such as, increase of the contact surface of the implant surface; favoring cell adhesion to the surface of the implant; increasing the amount of bone tissue with the surface of the implant; increased biomechanical interaction of bone with implant; inflammation of the peri-implant mucosa, if the rough surface were exposed to the buccal environment. Considering this review and other findings in the literature (20,21), it is possible to hypothesize that surface roughness is directly proportional to the area and the bone perimeter in contact with the implant surface, corroborating the results of this study, which showed higher mean roughness values (Ra), roughness measurements in cross-section, linear extension of bone implant contact in percentage (BIC%) and neoformed bone area (NBA%) for the AFS group.
The surface of the implants of the AFS group when evaluated at SEM showed a rougher topography with presence of micro-cavities surrounded by sharp micropeaks, similar to peaks and valleys, an image compatible with surfaces modified by subtraction process. This type of topography helps the bone deposition process, as demonstrated by the histometric analysis that showed higher BIC% and NBA% values for AFS when compared to MS, which in the topographic characterization by SEM showed to be a smooth surface with presence of machining residuals and small grooves, characteristic of the metal machining process.
Previously published studies (22-24) that employed SEM to evaluate surfaces modified by acid etching with and without fluoride ion deposition reported that the surface with fluoride ions had a more homogeneous surface, with smaller micro-peaks, but still with sharp edges. These topographic characteristics may be associated to the high reactivity of the fluorine ion, producing a coalescence of the peaks, through the chemical susceptibility of the titanium oxide to these ions.
In a previously published study (22) it was observed, at SEM, that surface modified by blasting was more heterogeneous when compared to the surface with addition of fluorine ions. However, implants containing fluoride ions on their surface presented important characteristics for the osseointegration process, such as higher calcium-phosphorus binding capacity on the surface, which could indicate an increased ability to react with calcified tissues, optimizing osseointegration.
The semi-quantitative analysis of the chemical composition of the surfaces modified by acid conditioning followed by addition of fluorine ions from this experimental study, performed by the x-ray energy dispersive spectrometer (EDX) system, showed higher peaks of Ti and O and Na, Cl and F in smaller peaks. In a previous study that used a similar methodology (25), it is suggested that such phenomenon occurred due to the modification by the immersion technique in solution containing fluoride ions to add a small amount of this element to the titanium surface. In this context, new analyses, such as biomechanical analysis by means of removal torque and resonance frequency measurements, microtomographic analysis and fluorochrome analysis, should be performed in order to ratify the results found in the present study.

CONCLUSION

In view of the results obtained it is possible to conclude that the surface modified by acid conditioning followed tby he addition of fluoride ions enhances the osseointegration process, even in the later periods of bone repair.

Conflicts of interest

The authors declare that they have no funding, financial relationships or conflicts of interest to disclose.

Author contributions

Conceptualization, Francisley Ávila Souza and Paulo Sérgio Perri de Carvalho; Formal analysis, Alex Sandro da Silva, Luara Teixeira Colombo and Henrique Hadad; Investigation, Ana Flávia Piquera Santos; Methodology, Alex Sandro da Silva, Luara Teixeira Colombo, Henrique Hadad, Ana Flávia Piquera Santos, Pier Paolo Poli and Eduardo Vedovatto; Project administration, Carlos Nelson Elias, Francisley Ávila Souza and Paulo Sérgio Perri de Carvalho; Software, Ana Flávia Piquera Santos and Pier Paolo Poli; Supervision, Carlos Nelson

Elias, Francisley Ávila Souza and Paulo Sérgio Perri de Carvalho; Validation, Pier Paolo Poli; Writing: original draft, Luara Teixeira Colombo and Henrique Hadad, review and editing, Francisley Ávila Souza.

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