In-vivo bone response to titanium screw implants anodized in sodium sulfate[1]

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ABSTRACT

PURPOSE: To evaluate the early bone response to a nanotextured dental implant treated with sodium sulfate (Na_2SO_4), using a rabbit model.

METHODS: Twelve animals were randomly divided into group 1 (Control) – machined implants and group 2 (Test) – nanotextured implants. Extra-oral incision was performed to provide access to intended surgical site where the dental implant was inserted immediately after the extraction of the mandibular first premolar. Implant surface characterization was performed by scanning electron microscopy attached to energy dispersive spectroscopy and interferometry. Three weeks after surgery, the animals were induced to death and undecalcified sections of the samples were prepared for histological and histomorphometrical analysis.

RESULTS: Surface characterization of the implant demonstrated enhanced surface area of anodized group compared to Control group with 19.2% ± 6.2 versus 1.6 ± 0.7, respectively. Histological evaluation demonstrated new bone formation starting from the buccal and lingual cortical walls on both groups. After three weeks, significant higher bone contact of 27% ($p<0.05$) was observed to nanotextured compared to machined implants (Control group).

CONCLUSION: The anodization with sodium sulfate nanostructures to the implant surface that resulted in faster osseointegration.

Key words: Dental Implants. Nanotechnology. Osseointegration. Rabbits.

Introduction

After tooth extraction, changes in the alveolar bone and the healing process result in the formation of trabecular and medullary structures and significant bone loss is observed in the socket[1]. The goal of implant rehabilitation therapy is to preserve soft and hard supporting tissues, preventing continuous bone loss.

Thus, in recent decades there has been a substantial development of synthetic prostheses to replace or restore the functionality of tissues in humans, and, in the context of dentistry, commercially pure titanium has become popular due to its biocompatibility and mechanical properties. Titanium is, therefore, the gold standard for the purpose of comparison between different materials[2].

Modifications to the machined (also known as turned) implant design and electrochemical surface treatment (anodizing) of dental implants in the nanometer range (accurate to within 100 nm, i.e., one millionth of a millimeter or less) enhance adhesion, differentiation, and cell proliferation that ultimately results in faster osseointegration[3]. Enhanced osseointegration will improve anchorage and will provide larger surface area for distribution of occlusal load. Indeed, the development of a different nanomodified implants showed the desirable ability to form apatite layers in vitro nanotexturization[4], as well as good response to the living bone[5].

The investigation of healing events at the bone-implant interface would benefit from animal models with similar bone structure as found in human jaws. We have recently reported bone remodeling of rabbit sockets grafted with hydroxyapatite based a novel approach[6] on the rabbit tooth extraction socket model[7].

Since these Ti oxide films have presented promising results in laboratory tests, to study their performance in-vivo seems to be timely and useful.

Methods

The Ethics Committee for Animal Use of the Federal University of Rio de Janeiro (UFRJ) approved the *in-vivo* experimental procedures proposed (number 80/09), also carried out in accordance to the Care and Use of Laboratory Animals (National Institutes of Health) handbook rules.

Twelve adult male albino New Zealand rabbits (*Orictolagus cuniculus*) were housed under appropriated environmental conditions, exposed to 12 hours light-dark cycles with standard chow and water *ad libitum*. All animals were between 2.5 and 3.0 kg in weight. The experimental groups were randomly distributed (Control group - machined implants and Test group – nanotextured implants) and submitted to the same surgical procedure. The anesthesia was performed by intraperitoneal administration of ketamine (60 mg/kg weight) and xylazine (10 mg/kg weight) in association with local anesthesia using lidocaine 1%. Both superior maxilla and jaw were stabilized in open position and 5.0 cm-long extra-oral longitudinal incisions was done from the last molar to the labial commissure encompassing the masseter. Thus, intrasulcular incisions were done in the molars, having 5.0 mm in length mesially to the first molar, with visibility of the posterior teeth, to facilitate subsequent extraction. After mucoperiosteal detachment, the first molar on the left mandible was extracted using forceps. The installation of implants with primary stability was performed immediately after extraction of the tooth, followed by simple layer suture (Figure 1). Postoperative analgesia was performed using oral tramadol hydrochloride and subcutaneously with antibiotic enrofloxacin 0.1 ml/kg, 5% (single dose). At the end of a postoperative period of three weeks, the animals were painlessly euthanized with an anesthetic overdose of sodium pentothal for extraction of a 3 cm mandibular segment enclosing the implant.

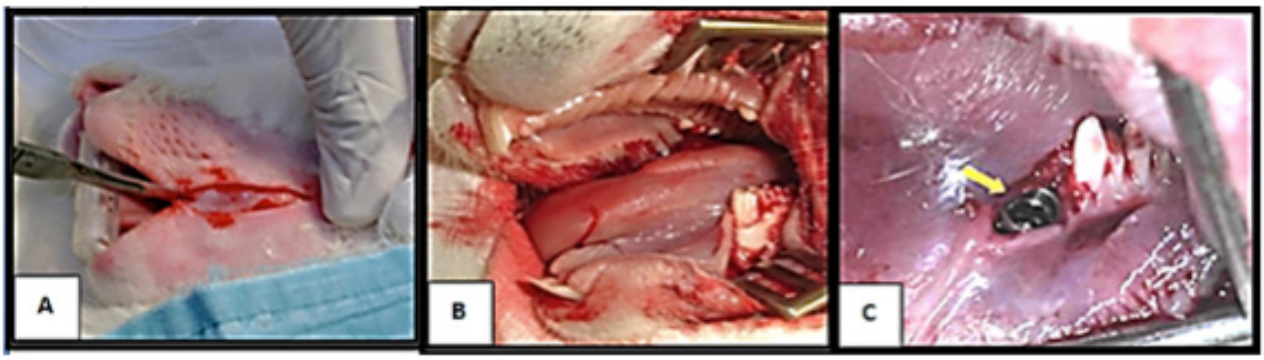

FIGURE 1 - Experimental surgery steps for screw implant insertion in rabbits' molar tooth: **(A)** incision to access the tooth; **(B)** exposing the tooth; **(C)** implant already inserted after molar extraction.

Surface treatment and characterization

Machined or turned commercially pure-titanium implants (INP Biomedical, Sao Paulo, Brazil) with dimensions of 4.0 x 8.0 mm² were submitted to surface modification by anodic oxidation (AO) process. Prior to AO treatment, the as-received samples were degreased in acetone ultrasonic bath for 15 min and dried in air. A circular Pt mesh (2.0 mm diameter) was employed as cathode where the Ti screws implants (anode) were gently centered inside. Then, the AO process was carried out at potentiostatic mode in 1.0 MNa_2SO_4 electrolyte under of 100V anodic voltage for 1 min.

Surface morphologies were observed by scanning electron microscopy (SEM, JEOL 6460 LV) working at 20 kV with an energy dispersive spectrometer (EDS) coupled to. Roughness analysis was performed by profilometry using an interferometer apparatus (NewView 7300, Zygo Corporation, USA) and scanning 200 μm x 260 μm areas with 0.4 μm and 0.05 nm lateral and vertical resolutions, respectively. Raw data were treated by using Gaussian filters, which is suitable for separating the shape and waviness of the measured roughness. Mean average height (Sa), developed surface area (Sdr) and local summits per area (Sds) were the main parameters measured.

Histological and histomorphometry evaluation

Extracted samples were fixed in 10% formalin solution at pH 7 for 10 days. Thus, the samples were placed in polyethylene vessels containing pure resin (LR White Hard Grade, UK), dehydrated in increasing alcohol solutions from 70% to 100% concentrations and replaced every 48 h. The specimens were kept in vacuum, stirred and stored at 4°C for nine days. After that, they were embedded in polytetrafluoroethylene (Teflon) molds and maintained at 60°C for resin polymerization. The polymerized pieces were halved and polished using an Exakt system (Norderstedt, Germany). They were cut in the buccal to lingual direction using a diamond saw tool. Surfaces containing implants were abraded using sandpapers until surfaces were smoothes. Then, the pieces were glued on an acrylic plate and their exposed surfaces were abraded and polished again to obtain 70 μm thicknesses, approximately. Finally, samples used for histological study were stained with both Stevenel's blue e Alizarin red markers and examined by optical microscopy (Eclipse 80i, Nikon, Japan) with x1 – x20 magnifications. Histomorphometry evaluations were carried out by image analysis using commercial software (Nikon Elements BR, Nikon, Japan).

Statistical analysis

Bone-implant contact results were statistically analyzed by the Wilcoxon method (Signed Rank Test), where p-value < 0.05 refers to significant difference.

Results

Surface characterization

SEM-EDS evaluation of machine implants (Control group) showed irregular surfaces with presence of micron-sized grooves and the chemical elements were titanium (Ti) and oxygen (O) (Figure 2A). Anodized implants revealed quite uneven porous surfaces embedded on a complex matrix with a wide range of pores size distribution. The qualitative EDS analysis also shows that the nanotextured implant surface was consisted of Ti, O, and the presence of traces of residual sodium (Na) and sulfur (S) derived from the electrolyte used in the anodization process (Figure 2B).

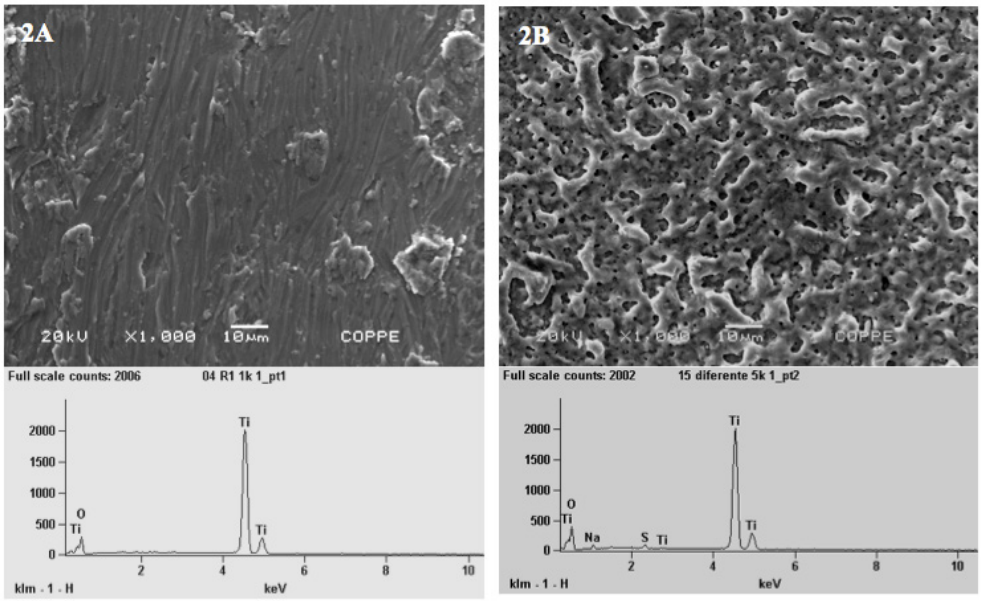

FIGURE 2 – SEM micrographies (x1.000) and EDS spectra of the machined or turned implants **(2A)** and treated Ti screw or anodized implants **(2B)**. The surface of the Control group **(2A)** was irregular with micron-size grooves with presence of Ti and O (Graphic attached to Figure 2A). The Test group **(2B)** showed micro pores and nanopores and the nanotexturized implant surface was consisted of Ti, O and a small quantities of residual sodium (Na) and sulfur (S) (Graphic attached to Figure 2B).

3D analysis of the surfaces images of the machined implants by interferometer revealed the presence of a more flattened surface, but with grooves and micro-fissures. The surface images of the nanotextured implant had a porous appearance with diffuse roughness (Figure 3). Data obtained from both groups were used for roughness parameters determination.

No difference was detected for both average height deviation (Sa) and density of summits (Sds) parameters. However, surface area (Sdr) of nanotextured implants was significantly larger than that of the turned implants ($p<0.01$).

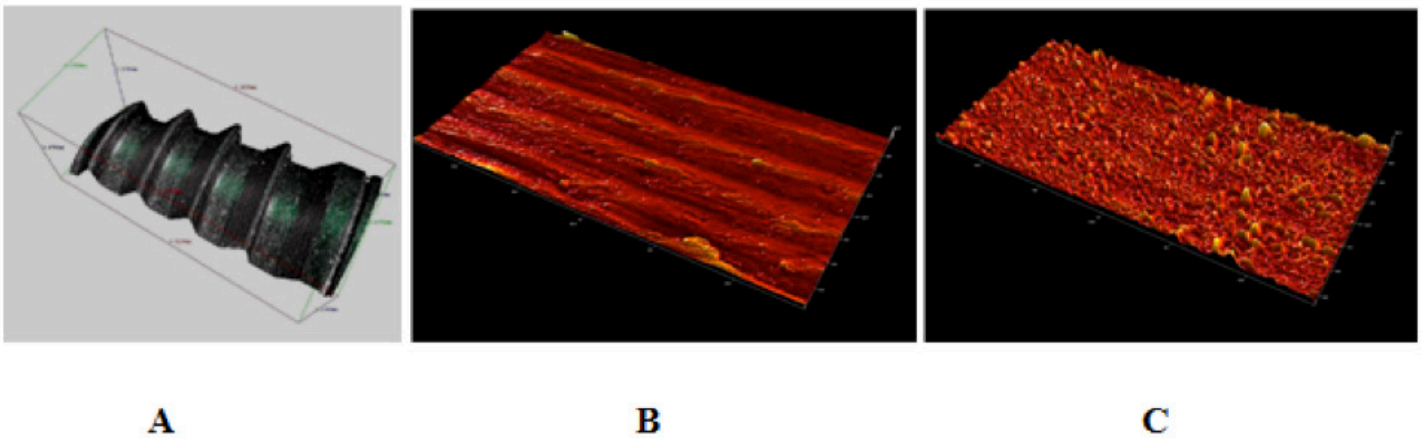

FIGURE 3 - 3D surface images obtained by profilometry: **(A)** 3D image of machined screw implant; **(B)** machined screw implant surface; and **(C)** anodized screw implant surface. Data obtained from B and C were used for roughness parameters determination.

Histology and histomorphometry

All implants were stable and partially covered by a soft tissue. Osseointegration was observed in all implants placed, with the presence of active osteoblasts and osteocytes in new bone matrix. Dental implant placed inside the rabbit socket presented new bone formation on the buccal and lingual walls of the socket. Moreover, such bone formation was found on the apex of the implant, inside the gap limited by the bottom of the defect and the implant. At higher magnification, closer apposition of newly formed bone was observed on the nanotextured samples (Test group) in contrast to the machined one (Control) (Figure 4).

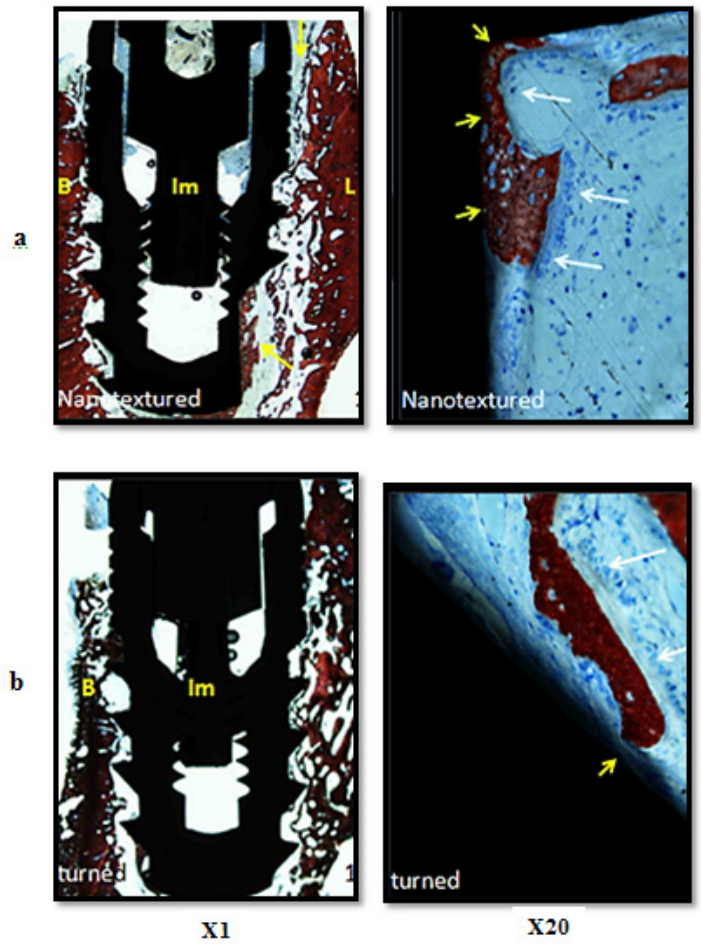

FIGURE 4 - Optical images of the histological evaluation of the healing process around the **(a)** machined/Control and **(b)** turned/treated implants. B – buccal wall; L -lingual wall, Im - Implant (x1 magnification). Yellow arrows indicate the contact area between the implants surface and newly formed bone tissue. Osteoblasts activities (stained in blue, white arrows) and bone-implant contact (short yellow arrows) were observed at higher magnification (x20).

Histomorphometry analysis of the bone contact showed an enhanced bone formation when nanotextured samples (19.4 ± 7.2%) were compared to turned (14.2 ± 5.9%) implants (p=0.46) (Figure 5).

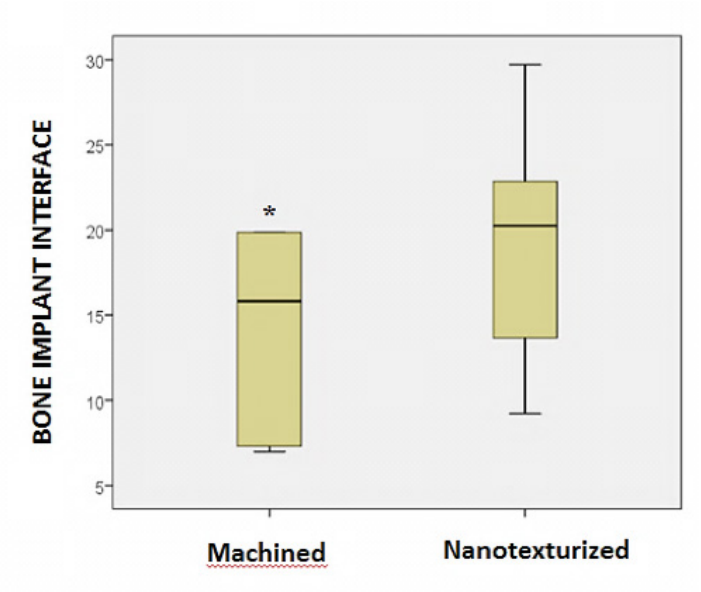

FIGURE 5 - Bone implant interface was lager in nanotexturized (Test group) than in machined (Control group) implants (*p<0.05).

Discussion

For this study, we used the rabbit taking into account the anatomical features of the mandibular structure, the compatibility of the model with studies of implants and also the applicability to human beings[9].

A recent systematic review about *in-vivo* tests found that the insertion of dental implants with intra-oral access is mainly limited to animals as large as dogs, monkeys, sheeps and pigs[10]. Additionally, *in-vivo* studies using rabbits, are usually carried out in tibia or femur[11,12]. However, such usual models impose several restrictions on translational studies, since these bones have different origins compared to the jaw (first arch) and possess a lower cortical-medullary thickness, thereby interfering with adequate bone-implant contact[13]. For a more realistic surgical applicability, the *in-vivo* model must include the tooth alveolus as an anatomical cavity. Indeed, as previously demonstrated by the authors[6], the rabbit implant socket is a feasible and successful *in vivo* model for the investigation of bone remodeling on fresh extraction sockets in smaller animals.

An essential step for mechanical stabilization of the implants in human beings is the sign of bone remodeling, which must have already appeared between the 8th and 12th weeks after implantation[14]. In our study, socket healing with formation of bone matrix was observed at the end of the third week. These results were similar to those obtained by Kurita *et al.*[15] and allowed for more rapid investigation of implant osseointegration. When the tooth alveolus anatomy is taken into account and a good surgical

technique used, dental implants promptly inserted after tooth extraction have shown excellent clinical success for the long-term use in both animals and human beings[16]. So this approach was followed in our experimental model.

For the surface analysis and topographic characterization of titanium implants in both groups, it was used the parameters of SEM and interferometer devices, suggested by Wennerberg *et al*.[17], whose properties complement each other. The advantage of SEM with EDS is its excellent depth of focus, which allowed high-definition imaging of the rough surfaces and established the chemical elements or contaminants present in the samples[18]. However a quantitative and 3D analysis of the topography was only achieved by interferometry[19]. The measurement of surface roughness parameters, based on a 3D analysis, showed a significant increase of the hybrid Sdr parameter to the nanotextured implant (19.19%) compared to machined one (1.55%), which means a large area at the surface treated with anodization.

Several studies into the application of dental implants have been developing continuously, with recent emphasis on nanotechnology[20]. This is a promising area for the generation of new prosthetic devices with bioactive surfaces, eventually with so-called smart surfaces, responsive to the local environment of implantation and promoting tissue regeneration[21].

The turned dental implant that contains minimal grooves on the surface is used as a standard when comparing surface treatments. Titanium anodic coatings prepared in 1.0M Na_2SO_4 (100V) have a rutile-rich crystalline structure, presenting rough surface with interconnected micro and nanopores[22]. So, the anodizing is an electrochemical process used to coat a metallic surface with nanoparticles and can turn the Ti surfaces of the implants into osteoconductive materials. In this way, the activity of mesenchymal cells and osteoblasts are facilitated during the bone remodeling. The osteoblast cells are attached on the surface by interposition of a tissue that interweaves into surface irregularities of the implant by biochemical and mechanical adhesion processes[23,24].

The surface treatment of cp-Ti screw implants with sodium sulfate (Na_2SO_4), as proposed in this study, led to forming Ti oxide coatings (1.6µm thick) with a rough and micron-sized porous layer on its surface with interconnected pores. The presented roughness can play an important role on exposing a larger surface to the biological medium, while the pores are able to anchor the osteoblast cell lamellipodium, as previously observed by Santos *et al*.[4]. Besides the better osteoblasts adherence on the TiO_2 coatings with Na_2SO_4 electrolyte, such surface also improves osteoblast proliferation[4].

Studies of patients treated with anodized implants in the mandible and maxilla revealed, respectively, a greater contact with the adjacent bone tissue than that observed in implants with turned surfaces, after mean healing periods of 6 ½ months and 3 ½ months[25]. In nanotextured implants (test group), the presence of a larger area of bone-implant contact and changes in the chemical properties of the implants due to the incorporation of sodium and sulfate ions upon anodizing seem to be able to stimulate osseointegration.

A few number of studies concerned about Ti anodic oxidation in Na_2SO_4 solutions have been published[8] but reports of Na_2SO_4 type of anodizing for dental implants *in vivo* were not found in the scientific literature in this context.

Conclusion

The *in-vivo* experimental model by using titanium implants in molar socket of rabbits provided evidence that nanotexturization of the surface of the dental implants, anodized with sodium sulfate, promoted earlier osseointegration with an improved bone response than turned implants commercially available.

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