Evaluation of a Titanium Surface Treated with Hydroxyapatite Nanocrystals on Osteoblastic Cell Behavior: An In Vitro Study

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Purpose: *In the context of macrostructural and microstructural modifications to the design of dental implants, surface topography changes with different treatments have the purpose of accelerating bone formation. The aim of this study was to evaluate in vitro the influence of aggregated hydroxyapatite nanocrystals to surfaces treated with double acid etching (Nano) on osteoblastic cell behavior compared with a conventional double acid-etched surface (DE).* ***Materials and Methods:*** *Commercially pure Grade 4 titanium discs (6 × 2 mm) were selected, and both cell proliferation and viability were assessed at 24, 48, and 72 hours using Trypan blue vital dye and MTT, respectively. The expression of type I collagen and osteopontin on such surfaces was evaluated using ELISA. Immunostaining for fibronectin was also performed. Quantitative data were analyzed statistically using two-way analysis of variance (ANOVA) followed by Bonferroni post-test with a 5% significance level.* ***Results:*** *The results showed that in all evaluated time periods, cells expressed fibronectin on both surfaces. The cells presented greater morphologic spreading on the Nano surface when compared with the conventional DE surface in all assessed times. Increased cell proliferation and viability were detected in the Nano surface ($P < .05$) when compared with the conventional DE surface, especially after 72 hours. Osteopontin expression was higher after 24 hours in the Nano surface when compared with the conventional DE surface ($P < .05$). For type I collagen, a higher expression was observed with the Nano surface than with the DE surface, again after 72 hours ($P < .05$).* ***Conclusion:*** *This in vitro study showed that the treated Nano surface tested promoted increased cell proliferation and viability when compared with the control surface. Additionally, increased cell spreading as well as type I collagen and osteopontin secretion were observed, favoring the early events of osseointegration.* Int J Oral Maxillofac Implants 2017 (6 pages). doi: 10.11607/jomi.5887

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Based on the principle of osseointegration, implant surfaces in direct contact with vital hard and soft tissues must be biocompatible and present adequate mechanical properties.[1]

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The characteristics of an implant surface include morphologic, physical, chemical, and functional changes that may directly influence the behavior of cells in terms of adhesion, proliferation, and differentiation. The main aspects relating to implant surfaces are topography, chemical composition, surface charge, and surface energy,[2] modifications of which may have an impact on cell interactions with the surrounding tissues.[3]

The various methods used to modify the topographic features of implant surfaces aim to yield uneven surfaces, improve the shear strength of the metal, thus providing better mechanical stability as well as promoting increased bone-to-implant contact (BIC), osteoblast proliferation,[4] expression of osteoblast phenotype-related markers, and synthesis of mineralized matrix in vitro.[5]

Studies show that the combination of different surface treatment methods can be advantageous for mineralized matrix neoformation.[4,5] The interactions between bone matrix and cell-signaling pathways

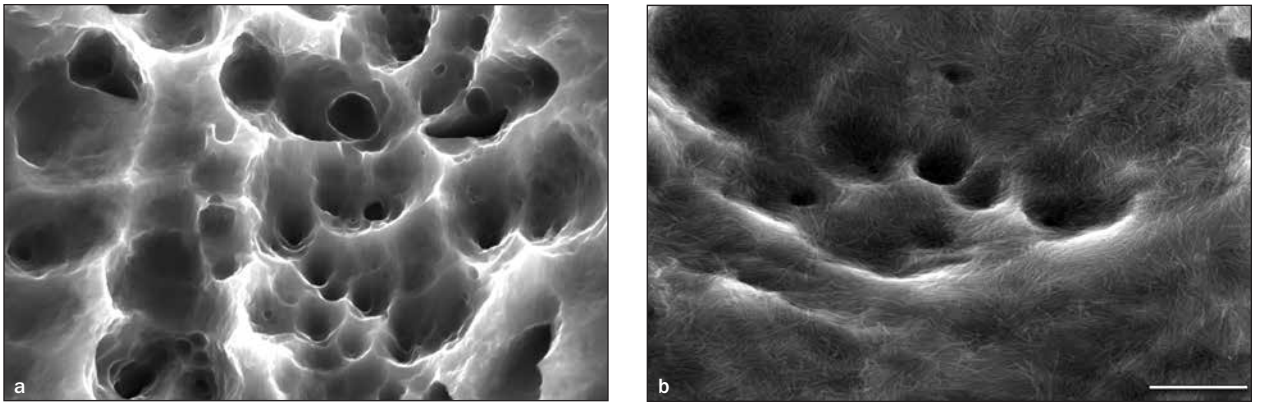


Fig 1 Representative scanning electron micrograph of the *(a)* double-etched and *(b)* hydroxyapatite nanocrystal-treated surfaces. Scale bar = 0.8 µm.

occur in Nano topographic scale.[6,7] Studies in vitro also show the advantage of combining surface treatments in terms of adhesion, proliferation, synthesis, and secretion of extracellular matrix.[6] Furthermore, the expression of osteogenic markers such as osteopontin (OPN) and bone sialoprotein (BSP), two major proteins of bone matrix responsible for biomineralization and bone remodeling,[8] significantly increase the Nano-treated titanium surface, suggesting an advantage of this type of surface treatment over others.[9]

Implants with hydroxyapatite (HA) coatings have been used to stimulate the osseointegration process. Such coatings are naturally highly osteoconductive[10] and have superior clinical longevity.[11] Long-term studies, however, show that treatment with plasma spray generates surface roughness ranging from 50 to 200 µm with a relative weak adhesion between the HA and the metal surface, which can therefore separate during the process of implant placement.[12–14]

To minimize possible losses of such a layer, the present study evaluated the early osseointegration events in vitro in the presence of a double acid-etched surface combined with HA nanocrystals as a uniform layer of 20 nm (Nano).

MATERIALS AND METHODS

Commercially pure titanium (grade 4) discs measuring 6 mm in diameter and 2 mm in thickness were used. The double-etched discs (n = 21) surfaces were aggregated with HA nanocrystals (Nano) to form a 20-nm-thick layer (Fig 1). For comparison, a double acid-etched surface (DE) (n = 21) was used, all of which was obtained from SIN. The ultrastructural morphology of these surfaces is represented in Fig 1 by scanning electron micrographs.

Preosteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC). The culture conditions were DMEM/F-12 medium (LGC Biotechnology) combined with bovine fetal serum at 10% (LGC Biotechnology) and penicillin at 100 U/mL plus streptomycin at 100 µg/mL (Sigma). The culture medium was replaced every 2 to 3 days, and the cell cultures were maintained in a moist incubator at 37°C containing 5% CO_2 and 95% air.

Fibronectin Immunolocalization

After 2, 4, 8, and 24 hours, culture fixation was performed in phosphate-buffered saline (PBS) formaldehyde at 4%, pH 7.2 for 1 hour at room temperature, followed by rinsing in PBS. The cells were then stained using 2% Alizarin red (Sigma), pH 4.2, for 10 minutes at room temperature. Cell permeabilization was achieved using 0.5% Triton X-100 in PBS for 10 minutes. Unspecific binding was blocked using a skimmed mild solution at 5% in PBS at room temperature for 30 minutes. Fibronectin expression was then ascertained using the monoclonal antibody anti-FN (1:300, Dako, Cytomation, Glostrup). The negative control consisted of PBS instead of the primary antibody. The secondary antibody was goat anti-rabbit conjugated with Alexa Fluor 488 (green fluorescence, 1:200, Molecular Probes). Excess antibody solution was discarded, and the samples were rinsed prior to mounting in Vectashield containing DAPI (4/-6-diamidino-2-phenylindole, Vector Laboratories). Qualitative evaluation was performed using a conventional Zeiss Axioskop 2 fluorescence microscope (Carl Zeiss MicroImaging) equipped with a 63× Plan Apochromatic 1.4NA and 100× Plan Apochromatic 1.4NA lenses (Carl Zeiss).

Cell Proliferation

Cell proliferation was ascertained by conventional cell counting. Briefly, the cells were enzymatically retrieved using a solution of 0.25% trypsin and 1mM ethylenediaminetetraacetic acid (EDTA) (Gibco) at 24, 48, and 72 hours of culture. Cell counting was performed manually using a hemocytometer (Hausser Scientific) and was reported as number of cells $\times 10^4$.

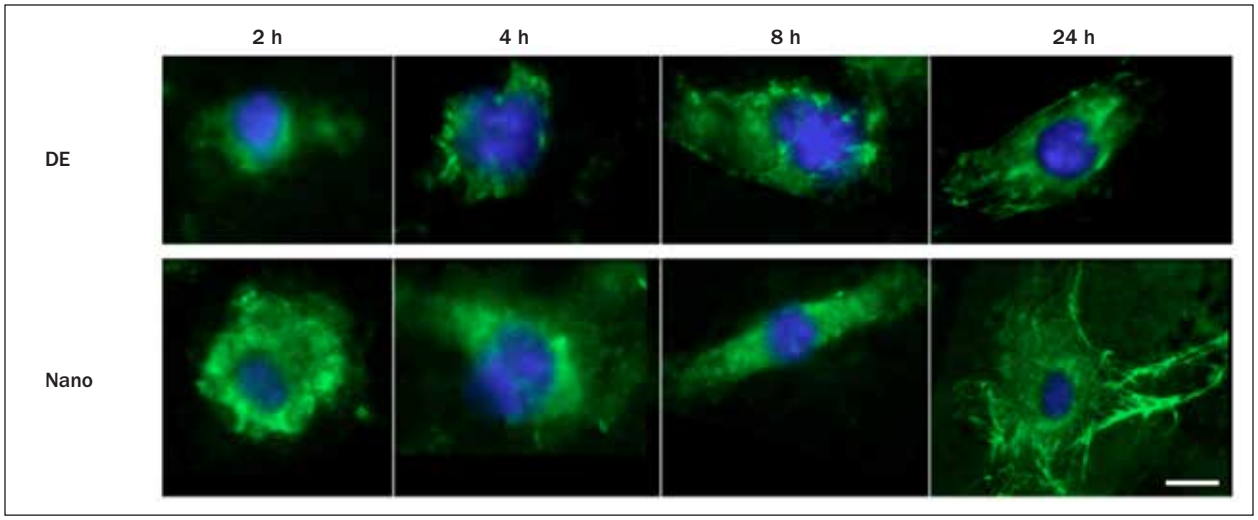


Fig 2 Immunolocalization of fibronectina (green, AlexaFluor 488) in MC3T3-E1 cells seeded onto DE and Nano surfaces after 2, 4, 8, and 24 hours. DNA staining (DAPI, blue). Scale bar = 50 µm.

MTT Assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT, Sigma) method at 24, 48, and 72 hours of culture. The cells were incubated with MTT at 10% in culture medium (5 mg/mL) at 37°C for 4 hours. The MTT solution was then discarded by aspiration, and 200 µL of Dimethyl Sulfoxide (Sigma) was added to each well followed by gentle agitation for 5 minutes on a plate shaker. Subsequently, 150 µL from each sample solution was transferred onto a fresh 96-well plate. Optical density was assessed at 570 to 650 nm on a spectrophotometer (Epoch; BioTek), and the data were expressed as absorbance.

Type I Collagen and Osteopontin Quantification using ELISA

Quantification of Type I Collagen and Osteopontin was evaluated at 24, 48, and 72 hours. Briefly, culture medium was collected from each sample and centrifuged at 336 g for 10 minutes, and the supernatant was aliquoted and stored at –80°C. Type I collagen quantification was performed using the Mouse Collagen Type I Kit (Wuxi Donglin Sci&Tech Development) and Mouse Osteopontin kit (R&D Systems) following the instructions provided by the manufacturer. The values were expressed as ng/mL.

Statistical Analysis

The experiments were performed in triplicate, and the data obtained were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's test at 95% significance.

RESULTS

Immunolocalization of Fibronectin

Representative images of fibronectin immunolocalization in cells seeded onto different surfaces are shown in Fig 2. In all evaluated times, the cells expressed fibronectin on both surfaces. The cells had a more widespread morphology on the Nano surface when compared with the DE surface.

Cell Proliferation Assay

The results showed that, with time, there is increased cell proliferation, especially after 72 hours, with significantly greater proliferation of osteoblastic cells on the Nano surface compared with the conventional DE surface (Fig 3).

Cell Viability Assay

The results showed increased cell viability in the Nano surface, especially after 72 hours ($P < .05$), when compared with the conventional DE surface (Fig 4).

ELISA

The results showed an increase of type I collagen expression on the Nano surface in relation to the conventional DE surface, especially after 72 hours ($P < .05$) (Fig 5a).

In 24 hours, an increased expression of osteopontin was observed (Fig 5b) in the Nanosurface when compared with the conventional DE surface ($P < .05$). After 48 hours, no significant difference was observed between the surfaces tested ($P > .05$).

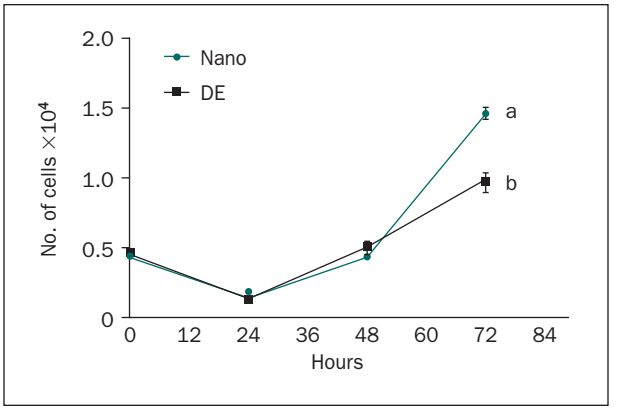


Fig 3 Cell proliferation according to the Trypan blue vital exclusion method in preosteoblastic cells (MC3T3-E1) at 24, 48, and 72 hours. The line chart represents the means and standard deviations from three separate experiments, which were statistically analyzed using two-way ANOVA post hoc Bonferroni. Different letters indicate a significant difference between groups ($P < .05$).

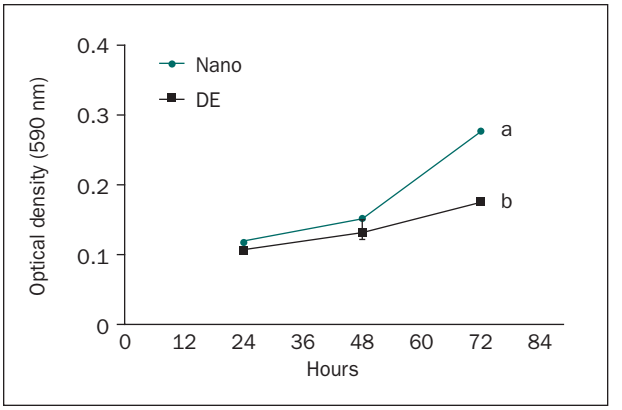
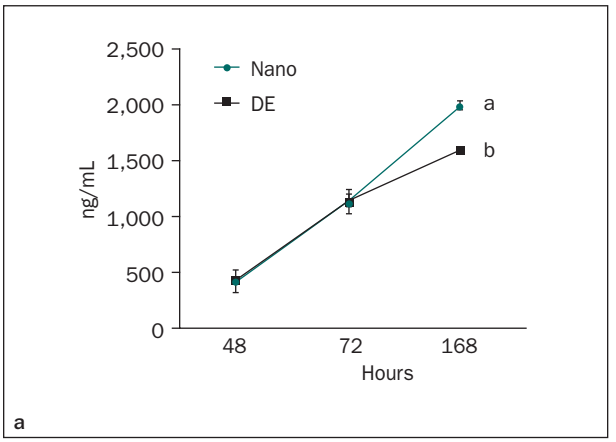


Fig 4 Cell viability assay in preosteoblasts (MC3T3-E1) in 24, 48, and 72 hours. The line chart is representative of an experiment run in triplicate. The values are expressed in means (± SD), which were statistically analyzed using two-way ANOVA post hoc Bonferroni. Different letters indicate a significant difference between groups ($P < .05$).



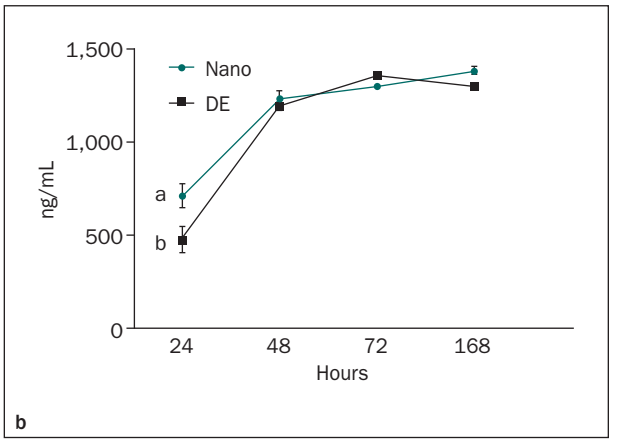


Fig 5 Quantification of *(a)* type I collagen and *(b)* osteopontin via ELISA. Data representative of the experiment run in triplicate. Values are expressed as mean (± SD). Two-way ANOVA followed by Bonferroni. Different letters indicate a significant difference between groups ($P < .05$).

DISCUSSION

In addition to modifications to macrogeometry and implant design, changes to physical and chemical characteristics of the implant surface have been recommended to accelerate bone response and promote faster osseointegration, thus reducing the risk of treatment failure. Among such modifications, surface roughness, surface energy, and the biomaterial can be highlighted. The present study evaluated the potential in vitro of a new surface treated by addition of a HA nanolayer to stimulate osteoblastic cells.

Surface treatments mainly cause increased roughness favoring adhesion of osteoblasts around the implant. Some studies in vivo have shown better quality and quantity of new bone formed around implants with treated surfaces with regard to promoting faster and greater cellular response and therefore adsorption molecules involved in the formation of mineralized extracellular matrix.[15–17]

Among the surface treatments, incorporation of inorganic components in the titanium oxide layer can also change the microscopic features of the implant. Authors have reported an increase in bioactivity of titanium alloys treated by the addition of nitrogen ions and an increase of cell adhesion and bone formation in implants treated by anodization methods associated with treatments with plasma spray and etching.[18,19] Furthermore, an increased cellular response has been associated with cpTi surfaces treated with biomimetic substances containing ions such as calcium, silicon, silver, and phosphorus by means of plasma oxidation. High levels of calcium and phosphorus and the addition of silver nanoparticles to the oxide layer have better surface properties as well as better cellular response.[20]

Over the past few years, studies in vivo have shown promising results regarding bone repair based on Nano topography titanium (Ti) implant surfaces.[21–23] According to some studies, implants treated with plasma-sprayed HA and acid etching present micrometric irregularities that promote better bone response compared with implants without the HA coating. Nevertheless, long-term studies on such implants show that treatment with plasma spray onto 50- to 200-µm rough surfaces suffers from weak adhesion between the HA and the metal, which can result in separation of the HA layer during implant placement.[12–14]

To minimize the possible loss of this surface layer, new types of thinner treatments have been proposed. Thus, in this study, a double acid-etched surface with an aggregated layer of HA nanocrystals and uniform thickness of 20 nm (Nano) was evaluated in vitro regarding its influence in the early events of osseointegration.

The results showed that when osteoblasts were grown onto the Nano surface, greater cell spreading, proliferation, and viability as well as increased expression of two proteins involved in the initial osseointegration process were observed when compared with a surface treated by double etching alone (DE).

Surface nanotopography plays an important role in various cellular responses,[24] since cell/matrix/substrate interactions associated with cell signaling occur at the nanometer level. Such signals regulate migration, proliferation, adhesion, and cell spreading, as well as differentiation and both gene and protein expression.[25,26]

A surface with a mean surface roughness (Ra) of approximately 1.5 µm induces a more significant bone response than smoother (Ra < 1 µm) or rougher surfaces (Ra > 2 µm).[27] The surface features evaluated in this study coated with a homogeneous 20-nm layer of HA nanocrystals had Ra values between 0.9 and 1.1 µm (unpublished data). This roughness favors the initial events in cell adhesion, proliferation, and cell spreading, as demonstrated in this study. It was observed that osteoblasts seeded onto Nano surfaces presented, within 24 hours, greater cell spreading when compared with the conventional DE surface. An increase in cell proliferation and viability was observed thereafter, which denotes stimulation of mitotic events, especially at 72 hours.

A recent study in vivo involving titanium surfaces treated with nano HA showed greater BIC and an increase in removal torque, suggesting that this type of material has satisfactory mechanical properties and collaborates toward high-quality bone formation.[28] In this context, collagen I and OPN are key proteins in the early events involved in mineralized matrix formation.

Collagen represents a major class of proteins present in the extracellular matrix of many connective tissues that feature, among many functions, a structural role. Type I collagen is described as an early marker of bone growth and osteoblastic differentiation and is present in the immature bone matrix secreted by osteoblasts.[29]

OPN is an important protein in the formation of noncollagenous bone matrix and is expressed by various cell types including osteoclasts and osteoblasts.[30] OPN also regulates angiogenesis as a response to cell stress, cell adhesion, chemotaxis, and cell motility.[31]

In this study, osteoblasts seeded onto the surface treated with HA nanocrystals (Nano) showed higher expression of type I collagen and OPN compared with the DE surface, especially after 72 and 24 hours, respectively.

It is important to consider that the HA layer applied to a rough surface may have influenced the phenotype and differentiation of osteoblasts. It is widely described in the literature that the optimal size of the pores to promote bone formation, allowing the organization at the microscopic level of the vascular system, varies between 100 and 500 nm.[32–34] Furthermore, the three-dimensional aspect of a Nano-treated surface mimics that seen in trabecular bone, thus increasing the surface area and promoting both cell proliferation and bone formation.[35] Viornery et al[36] evaluated osteoblast cultures over titanium discs modified with phosphoric acid and showed increased expression of type I collagen on the surfaces featuring nanopores (Ra values of 81 nm) compared with machined surfaces. Corroborating the results of this study, Dalby et al[37] and Lee et al[38] demonstrated greater OPN expression induced by HA in osteoblastic cells.

CONCLUSIONS

The results of this study in vitro showed that the double acid-etched surface aggregated with HA nanocrystals promoted increased cell proliferation and differentiation, contributing to the synthesis of bone matrix. Clinically, this surface meets the ideal requirements for rapid bone healing and osseointegration compared with surfaces free from aggregated HA nanocrystals, which together with the macrostructure of the implant, could translate into higher success of oral rehabilitation.

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