Effect of Bioactivation on Traditional Surfaces and Zirconium Nitride: Adhesion and Proliferation of Preosteoblastic Cells and Bacteria

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Purpose: The aim of this in vitro study was to reproduce and evaluate the response of bone and bacteria to traditional and innovative implant surfaces with different wettability. Materials and Methods: Two hundred fifty-two samples made of grade 4 titanium with different coating (machined [MAC]; double-etched, Ti-AE; zirconium nitride [Ti-ZrN]) were used for this in vitro study. Disks were divided into test (bioactivated using plasma of argon) and control group (untreated). To assess the surface morphology of the specimens, representative images were acquired via scanning electron microscopy (SEM). Murine preosteoblasts (MC3T3-E1) were used to study the biologic response in vitro, while the quantification of protein adsorption was achieved through the incubation of the titanium samples in a 2% solution of fetal bovine serum (FBS) in phosphate-buffered saline (PBS). The sterilized titanium disks were then colonized by bacterial species from a single sputum sample obtained from a healthy volunteer. For every analysis, 24 disks were used (12 for each group). Results: SEM and topographic analyses demonstrated a Sa value of 0.33 (Ti-ZrN), 0.34 (MAC), and 0.62 (Ti-AE). Compared with the control groups, plasma treatment significantly increased the protein adsorption level on all the different titanium surfaces (5.88 ± 0.21 vs 7.85 ± 0.21, 7.13 ± 0.14 vs 9.74 ± 0.65, 4.41 ± 0.62 vs 6.13 ± 0.52, respectively, for MAC, Ti-treated, and Ti-ZrN). Similar behavior was described for cell adhesion (27.67 ± 2.03 vs 58.00 ± 20.13, 116.67 ± 12.02 vs 159.33 ± 8.09, 52.00 ± 4.73 vs 78.33 ± 4.67, respectively, for MAC, Ti-treated, and Ti-ZrN). Plasma treatment significantly augmented the number of CFU only in MAC and ZrN samples. Conclusion: With the limitations of this in vitro study, the following conclusions could be drawn: (1) rough implant surfaces present a higher adhesion and proliferation of preosteoblastic cells and bacterial biofilm; (2) rough implant surfaces benefited the most by the plasma of argon treatment. Int J Oral Maxillofac Implants 2018;33:1247–1254. doi: 10.11607/jomi.6654

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From a clinical perspective, the collar portion was mostly described to be the key factor in peri-implant bone resorption. For a long period, immediately after smooth-surface implants by the Brånemark System, hybrid implants with a smooth collar and neck have been available on the market with good clinical outcomes.

In the last decade, the increase of the peri-implant disease rate around fully treated surfaces motivated researchers to analyze new surface configurations, with the aim to minimize bacterial adhesion to the implant components. In fact, physical surface treatments and the consequent surface roughness play a more relevant role than only chemical modifications.

To prevent bacterial adhesion to the implant collar, hydrophobic coatings or low hydrophilic materials were suggested. Dental implants made of zirconia have been used increasingly over the past decade, owing to their favorable biologic, mechanical, and corrosion properties. In fact, despite its lower osteoblast adhesion, it was demonstrated that zirconia seems to possess a surface less prone to early plaque retention than titanium.

At the same time, in vivo and in vitro, zirconium nitride coating was demonstrated to alter the microbial composition in the short-term and in the long-term exposure. Moreover, in a different in vitro study, zirconium nitride was shown to favor cellular attachment of human gingival fibroblasts. In addition, in vitro, zirconium nitride thin films were shown to render the titanium surface more bioactive for preosteoblasts than bare titanium itself. Enhancement of physical surface properties (increasing surface energy, hence, hydrophilicity) was deemed to positively affect early bone responses. In the last few years, application of plasma technology on titanium surfaces highlighted that this technology allows higher wettability than only chemical modifications. Physical surface modification (subgroups) either were treated with plasma of argon or received no treatment at all, according to a computer-generated randomization, following a study design reported previously (Fig 1).

**Sample Size**

A priori power analysis was prepared, referring to similar in vitro trials. Given that the true difference between the experimental mean and the control mean is 1.548, the study sample size is represented by 12 experimental specimens and 12 control samples so as to reject the null hypothesis that there is no difference between the mean values of experimental and control groups with a probability (power) equal to 0.95. The Type I error probability was set at .05. Twelve disks for each surface modification (subgroups) either were treated with plasma of argon or received no treatment at all, according to a computer-generated randomization, following a study design reported previously (Fig 1).

**Scanning Electron Microscopy**

To assess the surface morphology of the specimens, representative images were acquired via a scanning electron microscope (SEM) (EVO 50, Zeiss). Samples were manipulated as described elsewhere.

**Surface Roughness Analysis**

Area surface roughness parameters at different sites of the implant were obtained by SEM (EVO MA 10, Zeiss). In particular, the Stereo-SEM (SSEM) technique was used. This approach exploits the basic principle of stereo vision to turn conventional SEM images into 3D surface topography reconstructions based on specific software (Mex 6.0, Alicona Imaging). In the present analysis, SEM images used to build up stereo-pairs were obtained at 2,000. Roughness parameters according to ISO25178 were obtained from reconstructed images of an 80 × 110-µm area.

**Cell Adhesion**

Among the different osteoblast and preosteoblast cell models available, the preosteoblastic murine cell line MC3T3-E1 (ECACC) was selected for this in vitro study. Cells were maintained in and manipulated as reported in detail in previous papers. Briefly, a suitable number of cells were seeded on the different samples, and subsequently, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were thus acquired and cell nuclei were counted following established protocols.

**MATERIALS AND METHODS**

**Sample Preparation**

Two hundred fifty-two samples made of grade 4 titanium (Resista, Omegna [VB]) were machined to obtain 8 × 3-mm cylinders. Three different surfaces were attained. The term “machined titanium” (Ti MAC) will be used henceforth to refer to the uncoated samples. Rough surfaces were prepared starting from pristine titanium by dual acid etching (Ti-AE). Zirconium nitride thin film coating (in the following named Ti-ZrN) was grown on pristine titanium through radiofrequency–plasma enhanced chemical vapor deposition (RF-PECVD).

The samples were thus acquired and cell nuclei were counted with 4',6-diamidino-2-phenylindole (DAPI). Images were thus acquired and cell nuclei were counted following established protocols.
Protein Adsorption
The amount of protein adsorbed on the surfaces tested was quantified as reported elsewhere with a SERVA BCA Protein Assay Micro Kit (SERVA Electrophoresis).

Bacterial Biofilm Evaluation
The sterilized titanium disks were colonized by bacterial species from a single sputum sample obtained from one healthy volunteer. Bacteria were grown overnight in 10 mL of Mueller Hinton (MH) broth (Sigma Aldrich) at 37°C. The day after, bacteria were subcultured until a spectrophotometric density of 0.6 at 600 nm was reached, corresponding to approximately $1 \times 10^8$ colony-forming units (CFU)/mL. Each disc was incubated with 1 mL of MH broth (for negative control) or 1 mL of bacterial suspension in a 24-well plate by using a shaking rotator (80 rpm) at 37°C for 24 hours. To remove nonadherent bacteria, each disk was rinsed in sterile saline and vortexed for 10 seconds, six times. Disks were then transferred into a sterile plastic container with 1 mL saline solution and sonicated at 80 kHz with a power output of 250 W. Afterward, tenfold dilutions of each supernatant were incubated in MH plate for colony counting.

Statistical Analysis
GraphPad Software was used to analyze the data, which were obtained by at least three independent repetitions. As usual, the level of significance was set at $P < .05$.

RESULTS

Topography
The three surfaces that were researched are shown in Figs 2 and 3. Machined (MAC) specimens display the characteristic marks left by the milling process (Figs 2a and 2b), which are still recognizable in the ZrN thin film–coated samples (Figs 2e and 2f). On the other hand, Ti-AE (Figs 2c and 2d) disks appeared as a typical roughened surface, as could be anticipated for the product of dual acid etching treatment.

Surface Roughness Analysis
Area roughness parameters obtained from the images are reported in Table 1. The different nature of surface roughness between the three sites can be better shown by average vertical roughness values (Sa and Sq). These values were near twice on the roughened sample compared with MAC and ZrN. Most importantly, the lateral roughness parameter Sdr, that is, the ratio between the actual surface area and the geometric area (in the present case, $110 \times 80 \mu m$), resulted in 51.24% in the roughened sample and 28.12% and 17.76%, respectively, in the MAC and ZrN samples. Data confirmed that the micro-rough topography obtained on acid etching provides a very significant increase of the implant surface area.

Protein Adsorption
As shown in Fig 4 and Table 2, plasma treatment significantly increased the protein adsorption level on
all titanium surfaces. Moreover, Ti-AE samples significantly increased the level of protein adsorption (7.13 vs 9.74) compared with Ti MAC (5.88 vs 7.85) and Ti-ZrN (4.41 vs 6.13) \((P = .0286)\).

Early Response of Osteoblasts

As depicted in Fig 5 and Table 3, on all the different titanium surfaces, plasma treatment significantly enhanced the cell adhesion level. Interestingly, both Ti-AE (116.67 vs 159.33) and Ti-ZrN (52.00 vs 78.33) samples significantly increased the MC3T3-E1 adhesion compared with Ti MAC (27.67 vs 58.00) \((P = .010)\).
Moreover, Ti-treated samples also strongly improved cell adhesion compared with Ti-ZrN (P = .0142).

### Bacterial Growth

In order to understand how plasma treatment of different Ti surfaces may affect bacterial growth, biofilm formation was evaluated at 24 hours recurring to CFU count. As reported in Fig 6 and Table 4, plasma treatment significantly augmented the number of CFU only in Ti MAC (1.1113 vs 2.1393) and Ti ZrN (1.3395 vs 2.0175) samples, but not in Ti-AE (2.7673 vs 2.2785). On the other hand, in the control group, Ti-AE samples presented significantly higher bacterial growth compared with Ti MAC and Ti-ZrN samples (P = .0167).

Absence of differences in the Ti-AE group after plasma treatment might be due to a saturation effect.

*Fig 4* Protein adsorption assay performed incubating samples with 2% FBS in PBS for 30 minutes at 37°C. Values represent mean ± standard error; the symbol (*) indicates a statistically significant difference, considering a P value < .05.

*Fig 5* Cell adhesion. MC3T3-E1 adhesion was evaluated on all samples at 20 minutes. The level of cell adhesion was measured counting the number of adherent cells for each field. Values represent mean ± standard error; the symbol (*) indicates a statistically significant difference, considering a P value < .05.

*Fig 6* Evaluation of CFU of bacterial biofilm growth on Ti disks incubated in 1 mL of bacterial suspension for 24 hours at 37°C in shaking. Values represent mean ± standard error; the symbol (*) indicates a statistically significant difference, considering a P value < .05.
DISCUSSION

The present in vitro study investigated the effect of bioactivation through plasma of argon on different implant surfaces. All activated surfaces demonstrated an increased affinity to cells and proteins involved in osseointegration (protein adsorption and osteoblast-like cell adhesion), but also to bacteria.

Although implant-supported rehabilitations were described to have a minimal failure rate, in critical anatomical (elderly people, patients with altered osseointegration processes) or surgical (immediate loading, implants inserted in grafted sites, short implants, low quality or quantity of bone) conditions, implants might present an increased failure rate.34–36

In these conditions, a more reactive surface would be helpful, increasing bone-to-implant contact and ensuring faster and stronger osseointegration. Research was highly focused on this field, developing the so-called “osseoconductive” surfaces.37

However, reports of increasing peri-implant disease incidence around fully acid-etched-, hydroxyapatite-, or titanium plasma spray–treated implants might suggest that a osseoconductive surface near the implant-abutment connection could be more prone to biologic complications following bacterial biofilm growth compared with smooth-surfaced implants.

To prevent any possible microbiologic disadvantages and exploit osseoconductive properties, hybrid-surfaced implants were brought on the market. This generation of implants was designed, in fact, with a rough surface in the apical portion of the implant and a smooth collar. The positive experience reported by machined and microtextured surfaces influenced the development of alternative surfaces, ideally capable of minimizing bacterial adhesion, including zirconium nitride.38,39

Although surfaces repellent for bacteria were also supposed to decrease affinity for bone-regenerative cells, zirconium nitride coating was demonstrated to be bioactive compared with smooth titanium.20 In the present study, protein adsorption tests demonstrated that the smooth surface behaved slightly better compared with Zr-Ni coating. On the other hand, this material showed a slightly higher cell adhesion compared with smooth titanium disks. Obviously, the dual acid-etched surface presented significantly better outcomes in terms of cell adhesion and protein adsorption. This behavior is likely correlated with the higher contact area available to cells and proteins.

Analyzing microbiologic outcomes, focusing on the control conditions (Ti MAC), data reported an increase of biofilm on the rough surface that is significantly higher compared with smooth surface and zirconium nitride. This is in accordance with the already-published literature, which reports how these surfaces demonstrated less microbiologic affinity compared with rougher micro-geographies.18,19

While a bioactivation treatment might increase the titanium reactivity to the bone environment, it also seems to increase the affinity to bacterial biofilm. In the present study, in fact, plasma treatment increased more than 2 times the bacterial growth on machined disks and almost 1.5 on zirconium nitride. Surprisingly, bioactivation decreased the bacterial growth on the studied rough surface. However, this might be due to a saturation effect. Additionally, large standard deviation values might suggest that microbiologic contamination values should be interpreted with caution.

In fact, while intergroup comparison demonstrated significant differences in the control condition with slightly better performance of machined and zirconium nitride surfaces, this difference disappeared when analyzing the intergroup comparison at the test conditions, although zirconium nitride presented a slightly better outcome.

However, the phenomenon might be clinically irrelevant. In fact, these surfaces were ideally created to be in contact at the time of the implant insertion with bone or connective tissues. Only an incorrect implant positioning might expose surfaces to the contaminated oral environment. Additionally, it must be highlighted that bioactivation through plasma of argon has a time-dependent effect. As demonstrated by Canullo et al.,40 the favorable influence of this treatment dramatically decreases in 48 hours. While it represents a control factor, this seems to be clinically relevant during the soft and hard tissue healing processes and the physiologic protein cascade, which are highest in the first 48 hours.41

However, data described in the present study might recommend the clinical advantage of a hybrid-surfaced implant (apical portion with a titanium rough surface to increase osseointegration and coronal portion with a smooth surface or zirconium nitride coating). At the same time, positive outcomes reported in the test group might suggest that plasma of argon treatment could exploit this hybrid surface configuration.

To better simulate clinical conditions and better generalize the clinical relevance, low concentrations of factors involved in the osseointegration process (proteins in bovine fetal serum and murine preosteoblasts) were used in the present in vitro study. In fact, based on previous experiments,21,23,42,43 higher numbers of cells or protein amounts were found to conceal the effect of plasma of argon, due to a saturation effect.40

To explain the results with a statistically significant increase of protein, cells, and bacteria after test conditions, the biophysical effect of plasma treatment on titanium surfaces should be clearly declared.
Bioactivation through plasma of argon, in fact, activates the electronic mantle of the external surface. This phenomenon produces an increasing surface energy, and therefore, hydrophilicity.  

Obviously, this activated status reflects on cell or protein interaction with titanium. With a similar biologic adhesion cascade, a similar influence was also proven to be active on bacteria.

Although this study was only in vitro and represents just a preliminary overview on the topic, the data obtained encourage the design and execution of clinical trials, in absence of which the relevance of the reported outcomes should be interpreted with caution.

**CONCLUSIONS**

Notwithstanding the limitations of this in vitro study, the following conclusions could be drawn. Rough implant surfaces present a higher adhesion and proliferation of preosteoblastic cells and bacterial biofilm. Rough implant surfaces benefited the most by the plasma of argon treatment.

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**REFERENCES**


