

Influence of Titanium Surface Treatments on Formation of the Blood Clot Extension

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The aim of this *in vitro* study was to evaluate the influence of 3 different implant surface treatments on the extension of human blood clot formation. For this purpose, the 3 types of surfaces (as-machined; test group 1, titanium discs blasted with aluminum oxide particles and washed with nitric acid; test group 2, titanium discs blasted with titanium oxide particles and washed with maleic acid) obtained were evaluated regarding topography and blood clot extension formation. Data suggest that different treatments applied on implant surfaces confer different mechanical and chemical properties, and that titanium discs blasted with aluminum oxide particles and washed with nitric acid exhibited the widest blood clot extension ($P < .001$).

Key Words: *implant, topography, surface treatment, blood clot extension*

INTRODUCTION

Peri-implant healing begins immediately after implant insertion by initial blood clot formation in the peri-implant gaps and the development of a layer of fibrins.¹⁻⁴ Blood clot or thrombus is the final product of the blood coagulation step in hemostasis. It is achieved via the aggregation of platelets that form a platelet plug and the activation of the humoral coagulation system (ie, clotting factors).

However, connective tissue cells, including osteogenic cells, do not come into direct contact with the implant surface. The peri-implant wound site is first occupied by a blood clot, which forms immediately around the implant. Connective tissue cells migrate through the remnants of the clot still attached to the implant surface, which has been modified by both ion and protein exchange.² The contact of blood with the implant surface triggers a cascade of outcomes, which begin with the adsorption of plasma or serum proteins and continues through to the recruitment and activation of cells.

The cellular elements such as polymorphonuclear granulocytes arrive about 10 minutes following blood contact.⁵ Contact with the blood stimulates healing by platelet

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activation and provides a transitory biological matrix through which osteogenic cells can migrate to the implant surface topography.⁶ This transitory matrix may harbor connective tissue cells that initiate wound contraction about the fifth day.⁷ The proliferation and differentiation of bone cells have been reported to be enhanced by the roughness of implant surface topography. Additionally, a series of coordinated events, including protein adsorption, proliferation, and deposition of bone tissue, are probably affected by the different topography surfaces.

Different implant surface topographies may influence not only the adhesion of proteins and cells but also the cellular metabolism, such as cell proliferation, differentiation, and extracellular matrix formation.⁶ The chemical and physical characteristics of implant surface topography, roughness, energy, and chemistry are also responsible for adjusting the cell growth and function.^{6,7}

Several studies have demonstrated higher removal torque values and percentage of bone-to-implant contact for micrometer scale surface roughness of dental implants when compared with as-machined surfaces.⁸ The main issue of these studies triggered the search of "ideal" implant surface characteristics. Several implant surface preparations have been developed, such as grit blasting, titanium plasma spraying, acid-etching, anodic oxidation, laser preparation, or combinations of these. The dental implant quality depends on the chemical, physical, mechanical, and topographic properties of the surface. These different properties interact among them and determine the activity of the cells close to the dental implant surface.

The sandblasted acid-etched implant surface topography is obtained by treating the commercially pure titanium implant with a spray of air and abrasive materials (aluminum oxide [Al₂O₃] or titanium oxide [TiO₂])

for a prescribed period of time and under controlled pressure. After that, this modified surface is attacked with acid solutions at different temperatures and for periods of time in order to remove any residue and to condition the blasted surface.

Thus, the ability of the implant surface to retain the fibrin net plays an important role in the osseointegration process. Therefore, the aim of this in vitro study is to evaluate the influence of different surface treatments on human blood clot extension.

MATERIAL AND METHODS

Implant surface topographies

In this study, 30 titanium discs measuring 5.0 mm in diameter and 3.0 mm in length and made of grade 4 titanium were obtained using 3 different treatments: as-machined (control group, M); test group 1 (TG1), titanium discs blasted with aluminum oxide (Al₂O₃) particles (100 μm) and washed with nitric acid (HNO₃) solution; and test group 2 (TG2), titanium discs blasted with titanium oxide (TiO₂) particles (50–100 μm) and washed with maleic acid (HO₂CCH₂CHOHCO₂H).

Atomic force microscopy (AFM) (PicoSPM I plus 2100 PicoScan Controller, Milenia Biotec GmbH, Bad Nauheim, Germany) was used for the surface topography analysis, in contact mode. The AFM scanned areas of 60 μm × 60 μm of each specimen. Imaging and roughness analysis were performed before the addition of human blood.

The measured parameters, such as the arithmetic average of all profile point absolute values, the root-mean-square of all point values, and the average absolute height values of the 5 highest peaks and the depths of the 5 deepest valleys were measured for each group.

Evaluation of fibrin clot extension

The specimens were identified in a blind manner, and each specimen was positioned

on the bottom of a well in a 24-well cell culture plate. Whole venous blood (10.0 mL) from a healthy, nonsmoker, female volunteer was collected with the use of a syringe, and 50 μ L were immediately dropped onto the flat surface of the specimens. Because no anticoagulants were used and since the blood clotting already starts with the first drop of blood, this first drop was discarded in order to avoid bias in the study protocol. The plate was kept in a humidifying chamber for 20 minutes at room temperature³ and after that, the specimens were washed with phosphate buffered saline (PBS) 3 times for 5 minutes. Then, the specimens were fixed in 1% formaldehyde phosphate buffered saline for 15 minutes, and after 3 washes of 5 minutes in PBS, were incubated for 10 minutes in PBS containing 0.02 M glycine followed by 3 washes in PBS. The specimens were dehydrated by immersing them for 10 minutes in each of the serially diluted (25%, 50%, 75%, 95%, and 100%) ethanol solutions.

The specimens were placed on coded metallic stubs and kept in a desiccator chamber with dehydrated silica gel inside it, at room temperature for 3 days. For scanning electronic microscopy (SEM) (Philips XL 20kV, Bal Tec, Scotia, NY), the specimens were gold sputtered (Bal-Tec SCD-050) for 120 seconds and analyzed using a microscope equipped with energy-dispersive spectrometer. For the clot extension analysis, backscattering microscopic images ($\times 40$) were selected.

Image analysis

Images were analyzed using the specific software (Image J 1.4o/java 1.6.0_07 software, Wayne Rasband, National Institutes of Health, <http://rsb.info.nih.gov/ij>). The human blood clot was delimited using backscattering. The images were calibrated using the pixel as unit, and thereafter the delimited areas were adjusted to the threshold and the measuring tool allowed the percentage of clot area extension on the specimen surface

to be determined. The software automatically measured and summarized the area of the black points. A blinded examiner performed all analyses twice.

Statistical analyses

The surface topography and clot extension values were measured per sample and averaged per group. The statistical analyses were performed using the Kruskal-Wallis ($\alpha = .001$) test, and the Dunn test was applied for the pair-wise comparisons as posttest.

RESULTS

Implant surface topographies

Although the SEM images of specimens revealed clear differences of M specimens, when compared with TG1 and TG2 specimens that exhibited irregular surfaces (Figure 1), AFM showed substantial differences ($P < .05$) between these last groups. The AFM allowed the analysis of surface roughness of specimens at the micrometer scale (Figure 2).

The as-machined surface (M) exhibited only grooves produced by the manufacturing instruments with peaks of about 1.3 μ m and roughness of 0.188 μ m. The specimens of TG1 exhibited a surface characterized by defined (clear) grooves, in which peaks reached 6.5 μ m, valleys with 1 μ m, and roughness of 0.945 nm. The specimens of TG2 exhibited surfaces with irregularities as well, but AFM revealed peaks with height of about 3.5 μ m and roughness of about 0.593 nm (Table).

Blood clot extension

The distribution of the clot extension adhered to the titanium surface is shown in Figures 3 and 4. TG1 presented higher means ($P < .001$) when compared with groups M and TG2, however, no differences were observed between M and TG2 ($P > .050$).

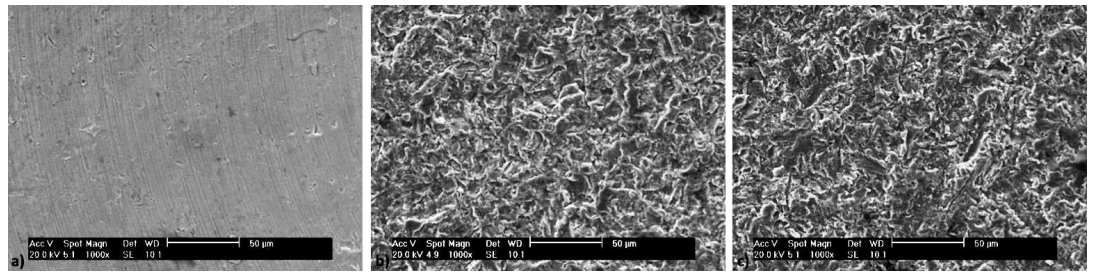


FIGURE 1. Scanning electron microscopy of (a) as-machined surface, (b) titanium discs blasted with aluminum oxide (Al_2O_3) particles ($100\ \mu\text{m}$) and washed with nitric acid (HNO_3) solution, and (c) titanium discs blasted with titanium oxide (TiO_2) particles ($50\text{--}100\ \mu\text{m}$) and washed with maleic acid ($\text{HO}_2\text{CCH}_2\text{CHOHCO}_2\text{H}$).

After exposure to blood, SEM images revealed blood elements such as fibrin, platelets, and erythrocytes on all groups.

DISCUSSION

This study evaluated the human blood clot extension on several implant surface topographies. The modified surface topography presents a geometric property that functions as a mechanical restriction for the cytoskeletal cell components that are involved in spreading and locomotion.¹⁰ Fibrin is originated from the reaction of thrombin and fibrinogen released into the healing site, and in dermal wound healing this process is followed by concomitant connective tissue cell migration and wound contraction. In the same way, this can occur at the peri-implant bony site, possibly causing retraction of the transitory fibrin scaffold away from the implant surface.¹¹ Thus, it appears that the wider the extension of clot retained on

implant surface, the better is the healing process and consequently, the osseointegration process.

Mechanical, chemical, and combined mechanical-chemical techniques are used to modify the surface roughness of materials. Grit-blasting or abrasive blasting are frequently used techniques for increasing the surface roughness of implant surfaces.¹² The mechanical treatments can originate surface roughness at 3 types of levels namely macro, micro, and nano.

Blood contact with proteins and, in our study, with different implant surface topographies, leads to the initiation of a clotting cascade via the intrinsic and extrinsic pathways, resulting in blood coagulation on the implant surface. Therefore, the activation of the coagulation system and platelets may have effects on cell and bone growth.^{4,13} Furthermore, several cytokines such as interleukin-1, platelet-derived growth factor, insulin-like growth factors, and vascular endo-

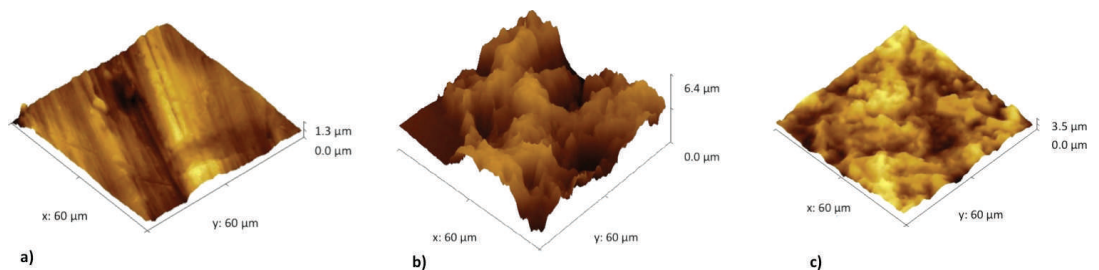


FIGURE 2. Atomic force microscope analysis of (a) as-machined surface, (b) titanium discs blasted with aluminum oxide (Al_2O_3) particles ($100\ \mu\text{m}$) and washed with nitric acid (HNO_3) solution, and (c) titanium discs blasted with titanium oxide (TiO_2) particles ($50\text{--}100\ \mu\text{m}$) and washed with maleic acid ($\text{HO}_2\text{CCH}_2\text{CHOHCO}_2\text{H}$).

TABLE

Mean and standard deviation of the as-machined (M) surface, titanium discs blasted with aluminum oxide particles and washed with nitric acid solution (TG1), and titanium discs blasted with titanium oxide particles and washed with maleic acid (TG2) profilometry†

Implant Surface Topography*	Ra, μm	Rq, μm	Rz, μm
M	0.14 ± 0.02	0.16 ± 0.01	1.61 ± 0.10
TG1	0.74 ± 0.07	0.95 ± 0.06	3.08 ± 0.94
TG2	0.48 ± 0.12	0.59 ± 0.21	2.09 ± 0.89

†Ra indicates arithmetic average of the absolute values of all profile points; Rq, the root-mean-square of the values of all points; Rz, the average value of the absolute heights of the 5 highest peaks and the depths of the 5 deepest valleys.

*Kruskal-Wallis test ($P < .05$). Statistically significant difference between the implant surface topographies ($P = .0001$), $M < TG2 < TG1$.

thelial growth factor are released from local cellular elements in the inflammatory phase and should enhance the wound healing at the implant site.

In this study, the combined mechanical-chemical approach was used to obtain the rough surfaces: M specimens were used as a control and these specimens were only machined/turned; TG1 specimens were subjected to airborne aluminum oxide particle abrasion (100 μm) and washed with nitric acid (HNO_3) solution; and TG2 specimens were subjected to airborne titanium oxide particle abrasion (50–100 μm) and washed with maleic acid ($\text{HO}_2\text{CCH}_2\text{CHOHCO}_2\text{H}$).

Moreover, differences were observed concerning the height and number of irregularities produced. This variability might be due to the difference in size (TG1: 100 μm ; TG2: 50–100 μm) and nature (TG1: Al_2O_3 ; TG2: TiO_2) of the material particles used for airborne particle abrasion and the different

nature of each acid used to wash the specimens (TG1: nitric acid; TG2: maleic acid). These chemical treatments can produce micropits with different sizes ranging from 0.5 to 0.2 μm in diameter.

Furthermore, it was observed that the TG2 surfaces exhibited a higher wettability when compared with those of TG1. In other words, when the blood was dropped onto the surface of TG2 specimens, it spread almost immediately, while on the surface of TG1 specimens the blood formed bubbles throughout most of the experiment (data not shown).

These wettability differences may also be due to the different acids used to manufacture the specimen. Specimens of TG2 exhibited lower peaks, and it appears that its wettability was more efficient.

The surfaces analyzed possess different topographies, which could lead to different biological responses such as mechanical interlocking in bone, cell adhesion, and cell

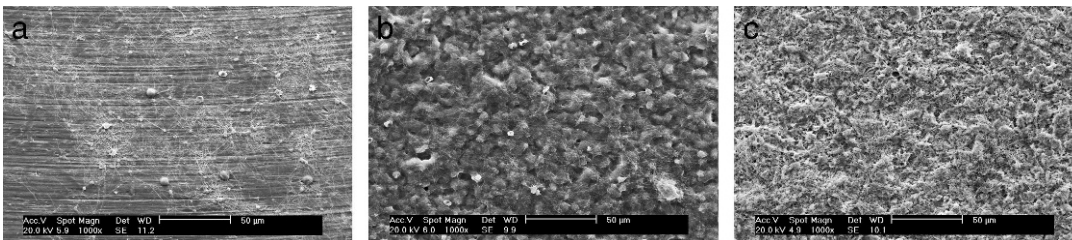


FIGURE 3. (a) Scanning electron microscopy of as-machined surface after 20 minutes of blood exposure. Fibrin filaments with trapped blood cells cover a small area of the implant surface. (b) Dense fibrin and red blood cells on TG1 covering all implant surface. (c) Fibrin scaffold is thin in TG2 surface. There are some implant surfaces exposed after blood exposure.

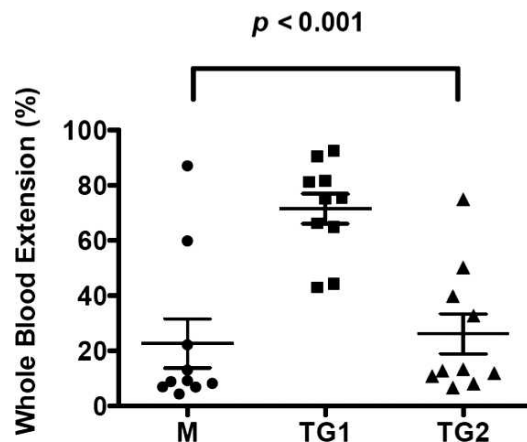


FIGURE 4. Box-plot of whole blood extension (% area) of all evaluated surfaces. Kruskal-Wallis test ($P < .001$).

morphology and orientation¹², bone neoformation¹⁴, enhancement of blood clotting,¹⁵ and cell differentiation.

The results demonstrated a wider extension of blood clot on the surfaces of TG1, which exhibited higher peaks and wider valleys when compared with TG2. Park and Davies¹⁶ also observed a higher agglomeration of blood elements on a micro-roughened surface when compared with a machined surface, which was also responsible for enhancing the platelet aggregation.

Although topography appears to be the main trait responsible for the clot extension adhered to the surface, another issue must be pointed out with regard to the different acids used during specimen processing. The different acids used might have generated different types of oxide layers, which could influence the adhesion of clot extension to the surface. TG1 group was washed with nitric acid, while TG2 specimens were washed with maleic acid, a compound with hydrophobic traits, which might influence the formation of blood clot extension.

CONCLUSION

Within the limits of this in vitro study, the data suggest that different treatments might generate surfaces with different mechanical

and chemical traits, which will exert an important role in the formation of blood clot extension. TG1 group presented the greatest clot extension ($P < .001$).

ABBREVIATIONS

AFM: atomic force microscopy
 PBS: phosphate buffered saline
 SEM: scanning electron microscopy

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