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ORIGINAL ARTICLE

Quantitative in vitro comparison of the thrombogenicity of commercial dental implants

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Abstract

Background: Dental implants often have surface modifications that alter surface topography and chemistry to improve osseointegration and thereby increase treatment predictability. Surface contact-induced blood coagulation is associated with the onset of osseointegration.

Purpose: To quantitatively evaluate the thrombogenicity of two commercially available dental implants that have similar surface roughness but different surface chemistry.

Material and Methods: Two commercially available dental implants with anodized or sandblasted acid-etched surfaces were evaluated for thrombogenic properties. Thrombogenicity was assessed by incubating implants for 1 hour in fresh, partially heparinized blood followed by hemocyte quantification, microscopic evaluation, and quantification of thrombogenic biomarkers.

Results: Fibrin coverage was significantly higher on the anodized surface compared with the sandblasted acid-etched surface ($P < 0.0001$). Platelet and white blood cell attachment followed a similar pattern. The increased thrombogenicity was confirmed based on a significant increase in the levels of the coagulation cascade biomarkers, thrombin antithrombin complex, and β-thromboglobulin (all $P < 0.05$).

Conclusion: Dental implants with comparable roughness but differing surface chemistry had differing extents of blood contact activation. These data suggest that surface chemistry from anodization augments implant thrombogenicity compared with that from sandblasting and acid-etching, which could have implications for osseointegration.

KEYWORDS

coagulation, dental implant, fibrin coverage, platelet activation, surface properties, thrombogenicity

1 | INTRODUCTION

To reduce early implant failure rates and allow the use of early loading protocols, current dental implants are designed for efficient osseointegration.[1] The first step in implant osseointegration is the coagulation of the patient's blood at the implant surface.[2] Indeed, fast and pronounced coagulation onset has been correlated with increased implant osseointegration in in vivo studies.[3–5] Blood, rather than osteogenic cells, is the first biological material to engage the implant surface.[5] When blood interacts with the implant surface, it forms a thrombus, which is a provisional fibrin mesh that traps myriad blood cells and biomolecules.[2] Thrombus formation is the first of several biological processes that drive bone healing. Next, the blood cells trapped in the thrombus release cytokines that recruit bone-forming cells to the implant surface.[6–9] Additionally, the fibrin mesh formed during coagulation presents ligands to which cells adhere, and stimulates cell proliferation and migration.[10] As osteogenic cells migrate through the fibrin mesh, they remodel and contract it.[11,12] To ensure that osteogenic cells reach the implant surface to deposit bone matrix, it is essential that the implant surface remains connected to the fibrin fibers during this cell-induced contraction.[2]

For this reason, extensive efforts have been directed towards the development of surface modifications that increase the induction of blood coagulation, that is, increase the thrombogenicity.[13] Several

physical and chemical surface features that potentially affect the surface thrombogenicity, such as implant geometry; macrotopography, microtopography, and nanotopography; implant material; surface oxidation; wettability; and charge, have been identified over the years.[14] In previous experimental studies, titanium discs were used to mimic the surface of dental implants, which allowed the study of a few surface parameters independently and shed light on blood-surface activation.[3,15,16] However, the thrombogenicity of a medical implant results from a combination of overarching parameters that should be considered simultaneously. To compare the thrombogenic potential of dental implants with different surface properties, it would be more predictive to use commercially available products rather than experimental discs. Moreover, model surfaces on experimental discs cannot account for the influence of packaging material, storage time, and implant geometry on coagulation. Further, experimental discs are not likely to be produced using the validated processes of commercial implant manufacturing, which could potentially lead to significant differences in surface properties and limit the value of study results. Thus, the effects of clinically relevant parameters, such as storage and packaging, are considered while conducting coagulation assays using commercially available dental implants.

Here, we compared the thrombogenicity of two commercially available high-performance surfaces: moderately rough anodized titanium (TiUnite, Nobel Biocare AB, Göteborg, Sweden) and sandblasted acid-etched titanium-zirconium alloy (ROXOLID, SLActive, Institut Straumann AG, Basel, Switzerland).

2 | MATERIALS AND METHODS

2.1 | Dental implants

Bone-level implants, ∅ 4.1 mm RC, SLActive 14 mm, ROXOLID, Loxim (REF 021.4314, LOT PK469), were purchased from Straumann AG, Basel, Switzerland. NobelParallel, Conical Connection RP, 4.3 × 13-mm implants (REF 37974, LOT 12108847) were provided by Nobel Biocare AB, Göteborg, Sweden.

2.2 | Contact-angle measurement

The dynamic water-contact angle of implants was determined using a Wilhelmy balance (DCAT21, DataPhysics Instruments GmbH, Filderstadt, Germany) and corresponding software (DCATS). Prior to measurement, all implants were rinsed twice for 120 seconds in 10 mL of ultrapure water and subsequently dried with a stream of nitrogen. The contact-angle determination data were acquired at five recordings per seconds with a descent rate of 0.1 mm/s until 10 mm of the implant was immersed. The subsequent ascent rate was 0.1 mm/s.

2.3 | Roughness measurement

Stack images of the implant surfaces were acquired using an Optical 3D Profilometer (S neox, Sensofar, Barcelona, Spain) using a 50× objective. Data were subsequently processed using the MountainsMap software (Digital Surf, Besançon, France) to determine the 3-dimensional surface roughness. Sa (arithmetical mean height) was determined after cropping a 100 × 100 μm area of the images and applying a polynomial 3 removal form and a gaussian filter (Filter Areal Linear Gaussian, ISO 16610-61) with an 80-μm cut-off.

2.4 | Blood incubation of dental implants

Whole blood from three healthy volunteer donors (ethical approval University Hospital Zurich, Switzerland: KEK-ZH 2012-0302) who had not used medication in the previous 2 weeks was drawn in 5 mL vacutainer tubes (Vacutainer No Additive (Z) Plus Tubes, BD, Switzerland) and supplemented with 150 μL of 16.7 IU/mL, 50 IU/mL, or 100 IU/mL heparin solution (B. Braun AG, Switzerland) to reach final concentrations of 0.5, 1.5, or 3 IU heparin per mL blood, respectively. Then, 1 mL of fresh blood (<1-hour old) was aliquoted into 1.5 mL cryotubes (Nunc, Thermo Fisher Scientific, Rochester, New York). Each dental implant was transferred into a cryotube using sterile forceps and incubated for 1 hour at 37°C. Tubes were flipped every 15 minutes to avoid blood sedimentation. After incubation, implants and blood were collected and analyzed as described below.

2.5 | Measurement of blood cells in blood

After incubation of dental implants in blood, 0.5 mL of blood samples were supplemented with 50 μL citrate, theophylline, adenosine, and dipyridamole (CTAD; BD, NJ, USA). A complete blood count was performed by the Haematology Institute of the University Hospital Zurich, Switzerland. Numbers of white blood cells, platelets, and red blood cells in blood were compared and reported as a percentage of the numbers in fresh blood (before incubation). Incubation of 1 mL of blood in an empty tube (blank) was used to determine the baseline cell consumption. For each surface type, two implants were incubated in blood from three donors, resulting in a total of six samples.

2.6 | Assessment coagulation cascade and platelet activation in blood

After incubation of dental implants in blood, 0.5 mL blood samples were supplemented with 50 μL CTAD to determine for enzyme-linked immunosorbent assays (ELISAs). Samples were immediately centrifuged at 2500*g* for 20 minutes at 4°C, then blood plasma was collected, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. Samples were assessed via ELISAs using commercial kits for β-thromboglobulin (β-TG; Asserachrom β-TG, Enzyme Immunoassay, Cat. 00950, Stago, NJ, USA) to evaluate the platelet activation and antithrombin (TAT; Enzygnost TAT Micro Test Kit, Cat. OWGMG15, Siemens-Healthcare, Germany) as a marker of coagulation cascade activation. ELISAs were performed according to the manufacturers' protocols. For each surface type, two implants were incubated in blood from three donors, resulting in a total of six samples.

2.7 | Fluorescence microscopy

After incubation of dental implants in blood, implants were rinsed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 minutes. Samples were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Missouri) in PBS. For the

detection of fibrin, samples were probed with primary mouse antibodies to fibrin(ogen) (F9902, 1:300 PBS; Sigma-Aldrich) for 2 hours at room temperature, followed by a 2-hour incubation with goat anti-mouse-FITC secondary antibody solution (1:300 in PBS containing 1% BSA; ab150113 Abcam, Cambridge, Massachusetts). Samples were also stained for actin (rhodamine phalloidin 1:500; R415, Invitrogen, Carlsbad, CA) and nuclei (Hoechst, 62 249, Thermo Fisher Scientific) and analyzed using fluorescence microscopy (DM550B, Leica Microsystems, Germany) or laser scanning confocal microscopy (SP5, Leica Microsystems, Germany). For each surface type, two implants were incubated in blood from three donors, resulting in a total of six samples.

2.8 | White blood cell quantification

To quantify white blood cells, blood-exposed implant surfaces were stained with a 1:1000 Hoechst 33342 solution in PBS for 15 minutes (Molecular Probes, The Netherlands). The samples were analyzed using fluorescence microscopy (DM550B, Leica Microsystems, Germany). For each surface type, two implants were incubated in blood from three donors, resulting in a total of six samples. For quantification, four images per sample were collected. Nuclei were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

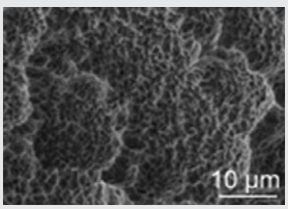
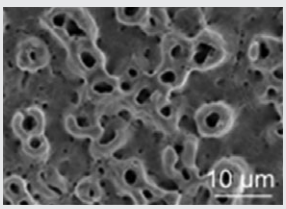
2.9 | Quantification of fibrin coverage

Fluorescence images were acquired using a DM550B microscope (Leica Microsystems, Germany). Fibrin coverage was evaluated by quantifying fibrin-stained areas using a constant manually set threshold in the ImageJ software. For each surface type, two implants were incubated in blood from three donors, resulting in a total of six samples. For quantification, four images per sample were collected.

2.10 | Scanning electron microscopy of adherent human blood components

After incubation in blood, dental implants were rinsed in PBS and fixed for 30 minutes in 2.5% glutaraldehyde in PBS. Samples were then dehydrated in a graded series of ethanol (from 25% to 100%). The samples were dried over the critical point of CO_2 (Tk = 31°C, Pk = 73.8 bar) using a critical-point dryer (CPD 030 Critical Point Dryer, Bal-Tec AG, Liechtenstein). The samples were sputter-coated with 5 nm platinum, and the images were recorded using a Leo 1530 scanning electron microscope (SEM; Zeiss, Germany) using a secondary electron detector and 10 kV acceleration voltage.

TABLE 1 Surface characteristics of implants used in this study

	Sandblasted acid-etched	Anodized
Manufacturer Catalogue Nr.	Institut Straumann AG REF 021.4314	Nobel Biocare AB REF 37974
Macroscopic images		
Diameter × length (mm)	4.1 × 14	4.3 × 13
Storage	Wet	Dry
Raw material	Titanium-zirconium alloy (ROXOLID)	Titanium
Microscopic images (scanning electron microscopy)		
Main surface elements[a]	Ti, C, O, Zr	Ti, C, O, P
Sa (µm)	2.1 ± 0.3	1.7 ± 0.1
Contact angle (°)	0	47 ± 5
Oxide layer thickness (nm)[a]	5-7	7000-10 000
Crystallinity of oxide layer[a]	Amorphous	Anatase-rich

[a] Data were extracted from Bernhard and colleagues[20] and Hall and colleagues[19] for sandblasted acid-etched and anodized, respectively.

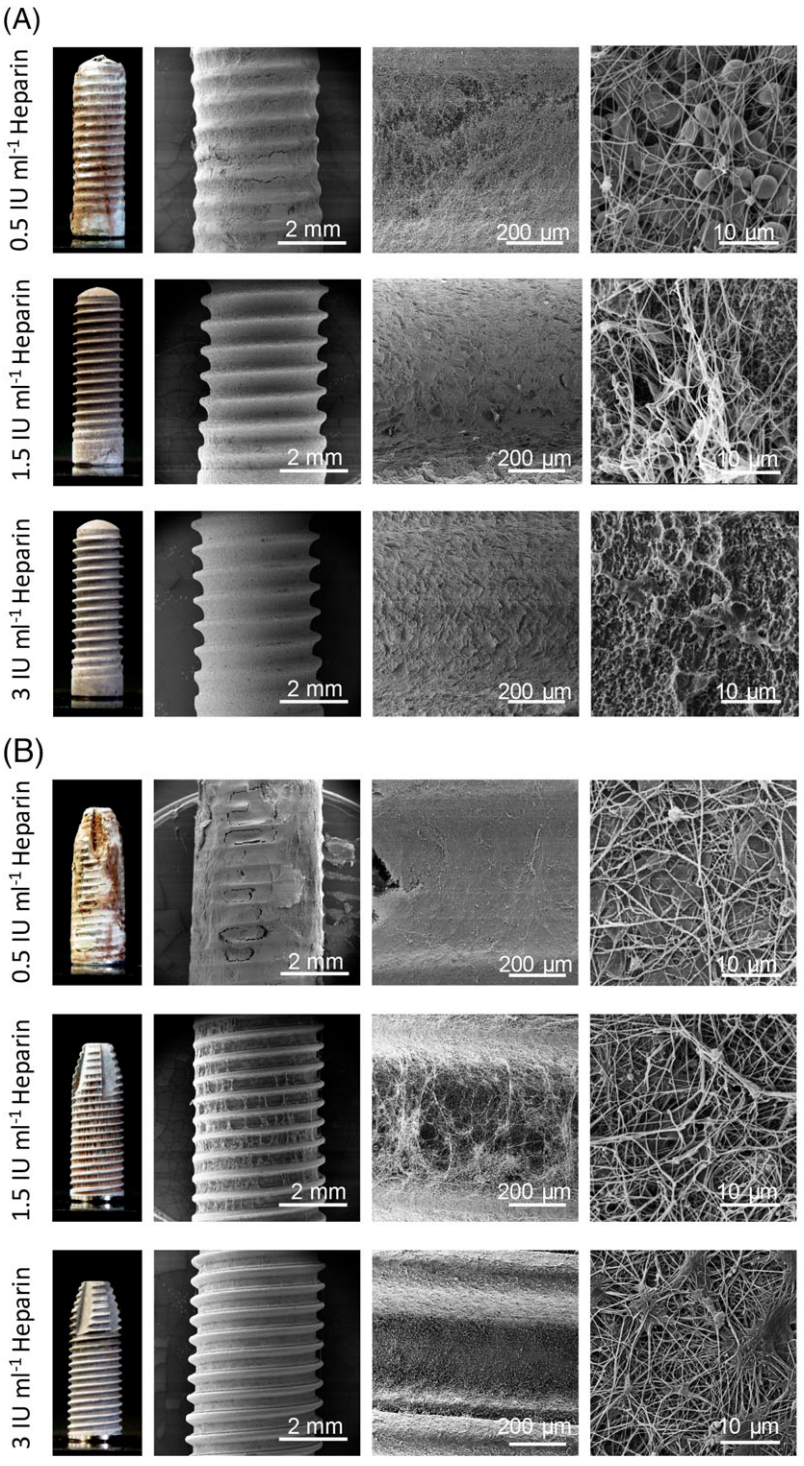


FIGURE 1 Assessment of thrombogenicity under varying heparin concentrations. Sandblasted acid-etched (A) and anodized (B) implants were incubated in 1 mL of human whole blood that was heparinized with 0.5, 1.5, or 3 IU/mL. Representative macroscopic and scanning electron microscopy images at various magnifications show the blood coagulation on the implant surfaces after 1 hour of incubation

2.11 | Statistics

Data are presented as mean ± SD. Mean values were compared using a Student's *t*-test. Statistical significance was designated as $P < 0.05$.

3 | RESULTS AND DISCUSSION

We investigated the thrombogenicity of dental implants with an anodized surface in dry storage (anodized surface) and a sandblasted acid-etched surface stored in a liquid container (sandblasted acid-etched surface). Both surfaces showed a similar surface roughness (Sa of 1.7 ± 0.1 µm for the anodized and 2.1 ± 0.3 µm for the sandblasted acid-etched), and are in a range that is reliable for promoting implant osseointegration (Table 1).[17,18] However, differences in raw materials, surface treatments, and storage conditions can lead to differences in the elements present on the surface, crystallinity and thickness of the titanium oxide layer, and hydrophilicity (Table 1).[19,20] For example, the sandblasted acid-etched surface had a contact angle of 0° and is

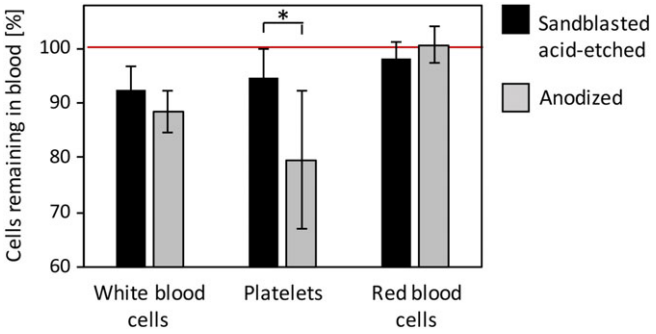


FIGURE 2 Quantification of blood cells following incubation with implants of different surfaces. Sandblasted acid-etched and anodized implants were incubated in 1 mL of human blood (from three donors) that was heparinized with 3 IU/mL. The number of white blood cells, platelets, and red blood cells remaining in the blood after 1 hour of incubation were counted. Red line represents numbers of white blood cells, platelets, and red blood cells in blood incubated with no implant. Data are mean ± SD (n = 6). * indicates P < 0.05 (Student's t-test)

considered to be ultra-hydrophilic. On the other hand, the anodized surface had a contact angle of 47° ± 5° and is thus, less hydrophilic.

To assess the thrombogenicity of the implant surfaces, coagulation was slowed in a dose-dependent manner with the anticoagulant, heparin.[21] We assessed the thrombogenicity both macroscopically and by using SEM. At a heparin concentration of 0.5 IU/mL, both implants were fully covered with a thick layer of coagulated blood after 1 hour of incubation. This confirms the strong thrombogenic potential of titanium surfaces, as reported previously.[3,5] To evaluate the difference in the early blood activation of each implant surface, heparin concentration was increased from 0.5 to 1.5 and 3 IU/mL, which is the range used previously in in vitro coagulation studies.[3,9,15,16]

The densities of the fibrin mesh and presence of blood cells on the two implant surfaces incubated with blood in 1.5 and 3 IU/mL heparin were different, both according to the macroscopic and SEM analyses (Figure 1). Surface-contact coagulation using 3 IU/mL heparin was further evaluated to perform a quantitative comparison of both implants.

Immediately after incubation, we assessed the number of white blood cells, platelets, and red blood cells that remained in the blood (Figure 2). The platelet count decreased to 95% ± 5% and 80% ± 13% for sandblasted acid-etched and anodized surfaces, respectively. Furthermore, the platelet count decreased significantly in blood incubated with the anodized surface compared with the sandblasted acid-etched surface (P = 0.02). No significant differences between the two surfaces were seen for white (P = 0.1) or red blood cells (P = 0.2). These observations were unsurprising because red blood cells are not a structural part of blood clots. In fact, a reduction of red blood cells would be indicative of hemolysis caused by improper handling techniques during the experiment.

To directly quantify the formation of the fibrin network and white blood cells adhering to the surface, we fluorescently labeled fibrin(ogen)—the building block of the fibrin meshwork—as well as cell nuclei and cytoskeleton (Figure 3A). After 1 hour of incubation, the fibrin network on the anodized surface was significantly more developed than that on the sandblasted acid-etched surface

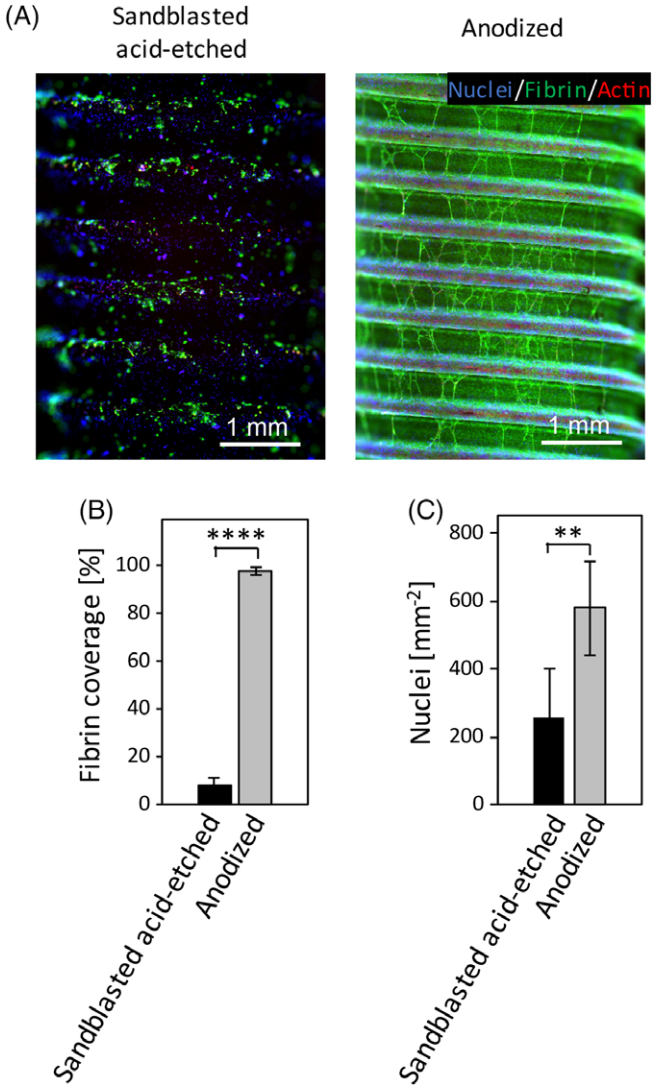


FIGURE 3 Fibrin polymerization and white blood cell attachment. Sandblasted acid-etched and anodized implants were incubated in 1 mL of human blood (from three donors) that was heparinized with 3 IU/mL. After 1 hour of incubation, blood coagulation was visualized by immunofluorescent staining of fibrin(ogen) and fluorescent staining with Hoechst (nuclei) and rhodamine phalloidin (actin). A, Representative low magnification fluorescence images of stained sandblasted acid-etched and anodized implants are shown. B, Quantification of fibrin coverage and nuclei present on implant surfaces. Data are mean ± SD (n = 6). ** indicates P < 0.01 and **** indicates P < 0.0001 (Student's t-test)

(P < 0.001, Figure 3B). Only 8% ± 3% of the sandblasted acid-etched surface was covered with a fibrin network whereas 97% ± 2% of the anodized surface was covered. Notably, fibrin fibers spanning from crest to crest of the implant threads were observed on the anodized implants, indicating advanced formation of the fibrin network. Additionally, 579 ± 137 fluorescently stained nuclei per mm^2 of white blood cells were observed on the anodized surface, which was significantly higher than that of the sandblasted acid-etched surface (257 ± 143 nuclei per mm^2, P < 0.001, Figure 3C). This difference was not apparent when analyzing the white blood cell count in post-incubation blood because the number of white blood cells in whole

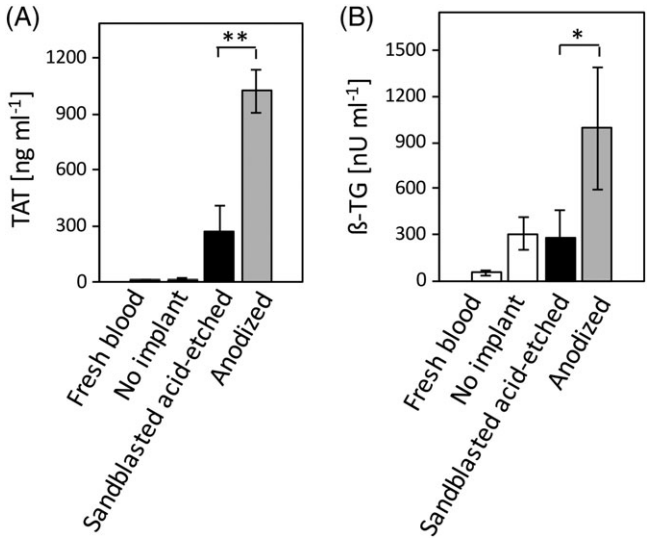


FIGURE 4 Coagulation cascade and platelet activation. Sandblasted acid-etched and anodized dental implants were incubated in 1 mL of human blood (from three donors) that was heparinized with 3 IU/mL. After 1 hour of incubation, thrombin formation (A) and platelet activation (B) in the remaining blood were quantified by performing ELISAs for thrombin antithrombin complex (TAT) and β-thromboglobulin (β-TG), respectively. TAT and β-TG levels in fresh blood and blood that was incubated without an implant were used as negative controls. Data are mean ± SD ($n = 6$). ** indicates $P < 0.01$ and * indicates $P < 0.05$ (Student's *t*-test). ELISAs, enzyme-linked immunosorbent assays

blood is 8×10^6 per 1 mL, and any differences would be too small to easily observe via cell counting.[2]

To further examine blood coagulation on the different surfaces, we measured the concentration of the thrombin TAT complex and β-TG, which are commonly used as measures of thrombin formation and platelet activation, respectively.[9] The average concentration of TAT complex in blood incubated with the anodized implant was significantly higher than that in blood incubated with the sandblasted acid-etched implant (1022 ± 114 vs 268 ± 142 ng/mL, $P = 0.002$, Figure 4A). TAT levels in fresh blood or blood that was incubated in the absence of an implant were 7.3 ± 2.5 and 14.6 ± 5.7 ng/mL, respectively, indicating that the increase in TAT levels was not due to the incubation step. β-TG levels of blood incubated in anodized implants was 992 ± 400 ng/mL and in sandblasted acid-etched implants was 282 ± 171 ng/mL. β-TG levels were significantly higher for anodized surfaces compared with sandblasted acid-etched surfaces ($P = 0.047$, Figure 4B). Interestingly, the β-TG levels of the sandblasted acid-etched implant were not higher than that of fresh blood (303 ± 105 ng/mL) or blood incubated without an implant (49 ± 12 ng/mL).

Coagulation assessments of partially heparinized blood in vitro are a broadly accepted assays to test early blood reactions at implant surfaces.[3,9,15,16] Here, we show noteworthy differences between two widely used implants. Given that the surface roughness of the two implants is comparable, the differences observed here are likely to be the result of the differing surface chemistries. For example, titanium anodization creates a highly crystalline, anatase-rich[19] surface with an increased surface charge,[22] and anodized surfaces have been shown to have more available hydroxyl groups[23] than acid-etched surfaces. A previous study correlated increased surface charge with increased thrombogenicity,[24] which could explain the results observed in this study. Moreover, metals incorporated with phosphorus were shown to promote osseointegration[25] and could also impact blood activation.

Given that an increase in thrombogenicity was reported to be beneficial for in vivo osseointegration, a more thrombogenic surface could possibly osseointegrate faster.[3–5] However, a patient's unique clinical situation will add additional complexity to the events taking place at the implant surface, and in vivo investigations are needed to clinically verify the impact of the current findings.

4 | CONCLUSIONS

The results of this study showed that an anodized implant surface stored dry is more thrombogenic than an acid-etched surface stored wet. This study showed that thrombogenicity is a multifactorial process dependent on surface chemistry. Taken together, surface anodization of titanium implants could create surface properties that are beneficial for coagulation. Because high thrombogenicity has been associated with faster implant osseointegration, these findings are consistent with the low failure rates and high predictability of immediate-loading protocols observed with anodized implants.

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CONFLICT OF INTEREST

P.S.L. and S.B. are employees of Nobel Biocare. V.M. became an employee of Nobel Biocare after completion of this study.

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