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Research Article

Comparative Analysis of the Influence of Titanium Surface Treatment with Different Acid Treatment Protocols on Bacterial Adhesion: an in Vitro Study

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Abstract

Background: Modifications in the surface of dental implants have demonstrated an important role in the optimization of osseointegration.

Objectives: The objective of this research was to evaluate in vitro if different titanium surfaces treatments promoted by acid solution at different times influence on the adhesion and viability of two bacterial species (S. aureus and S. mutans).

Materials and Methods: Commercially pure titanium discs of Grade 4 (6mm X 2mm) were treated with acid solution (hydrochloric, nitric and sulfuric) at 20 (P20) and 60 (P60) minutes, obtaining, respectively, mean roughness of 0.610 (± 0.037) µm and 0.773 (± 0.033) µm. As control, a machined surface not subjected to any treatment was used. In order to determine bacterial adhesion at different surfaces, the bacterial strains were cultivated in each sample at the density of 1X108 CFU/ml and incubated for 4 h at 37 ºC, in microaerophilic conditions. For quantification of live and dead adherent bacteria, the fluorescence technique with Live/Dead Baclight viability kit was used. The total area, as well as the area of live and dead bacteria were quantified by means of the Image program J. The statistical analysis was performed by means of Analysis of Variance followed by Tukey test, conducted at the significance level of 5%.

Results: For S. aureus, the Tukey test identified that the smallest counts and the smallest percentage of area were observed on the machined surface, while the highest values were found on the P20 surface. When considering the culture of S. mutans, the count and percentage of area were also lower on the machined surface, with no statistically significant difference between the values obtained P20 and P60 surfaces.

Conclusion: According to the results of the research, it was concluded that the microbial characteristics impacted the viability values. Despite the highest surface roughness of P60 surface, colonization of S.aureus was lower when compared to P20.

Keywords: Bacterial Adhesion; Dental Implant; Surface Roughness, Mucointegration; Fibrointegration

Introduction

Surface treatments of dental implants have demonstrated an important role in optimizing osseointegration, significantly changing concepts. Based on technological developments and studies based on immunohistochemistry and electron microscopy, it was established that these characterizations positively influence the behavior of osteoblastos [1-5]. However, the long-term prognosis of dental implants depends not only on osseointegration, but also on the quality of the seal between the mucosa and the implant abutment [6-8]. Studies have shown that final roughness, regardless of surface treatment, between 0.7um and 2.0um allowed direct adhesion of the osteoblast to the surface of the implants, while roughness lower than these values favored the adhesion of fibroblasts [9-12]. One of the critical points of implant rehabilitations is the gingival sealing around the prosthetic components. Unlike natural teeth, which have perpendicular fibers adhered to the dental tissue, implants only have circumferential fibers, as the vast majority of prosthetic components are machined, smooth, and do not allow fibers to adhere. For this reason, the maintenance of cervical margins is mainly due to the volume of gingival tissue, often requiring grafting procedures with connective tissue to prevent aesthetic or peri-implant health changes. Therefore, after the installation of dental implants, it is possible that pathogenic microorganisms from the oral cavity, in the sealing region between the gingival tissue and the implant platform, initiate gingival inflammation called mucositis, which can progress to cup-shaped bone resorption, characterizing the peri- implantitis, especially in individuals with poor oral hygiene [13-22]. In order to change the direction of the cervical gingival fibers, the treatment of prosthetic components has been considered and much discussed in the literature. With the consolidation of surface treatments for dental implants, literature has turned its attention to the sealing between the prosthetic component and gingival tissue. Published works have demonstrated that small roughness positively alters the behavior of collagen fibers. As most of these studies are still in vitro, bacterial behavior was not observed in relation to these treated prosthetic abutments and whether bacterial adhesion could somehow compromise the behavior of fibroblastos [23-28]. The objective of this work was to evaluate in vitro bacterial adhesion on titanium surface discs with roughness less than 0.7 µm, simulating the surface treatment on prosthetic abutments.

Materials and Methods

This work has received exemption from the Research Ethics Committee for Human Beings of the São Leopoldo Mandic Institute and Research Center, Campinas/SP as it is exclusively laboratory research, without the involvement of human beings or materials (Protocol 2016/06460, annex 1).

Samples: For this work, were used commercially pure grade 4 titanium discs (n = 18), 6 mm in diameter and 2 mm thick, supplied by the company Conexão Sistemas de Próteses (Arujá, São Paulo). For the treatment of the titanium discs, it was used a solution of sulfuric, nitric and hydrochloric acids, with times of 20 minutes (n = 6) and 60 minutes (n = 6). Discs without surface treatment called machined were used as a control (n = 6). Acid concentrations are not described as this is confidential company information. Roughness was measured using a contact profilometer (Mitutoyo, model Surftest SJ200, Brazil, Suzano). Four linear measurements were made on each sample in accordance with the DIN ISO 1302 standard, and the arithmetic average of the absolute values of each disc (Ra) was calculated. The average roughness (Ra) of the machined surface was 0.278 (± 0.035) µm, and after acid treatment, 0.610 (± 0.037) µm was obtained for a time of 20 minutes and 0.773 (± 0.033) µm for a time of 60 minutes. Figure 1 illustrates the morphological characteristics of the obtained surfaces. The discs were sterilized in ethylene oxide (Acecil, Campinas, São Paulo) and used in the following experiments. Figure 1 - Scanning Electronic Microscopy and Interferometry. A: machined surface, B: surface with 20 minutes of acid treatment, C: surface with 60 minutes of acid treatment. Original magnification: 15,000X. Detail insertion magnification of 70,000X.

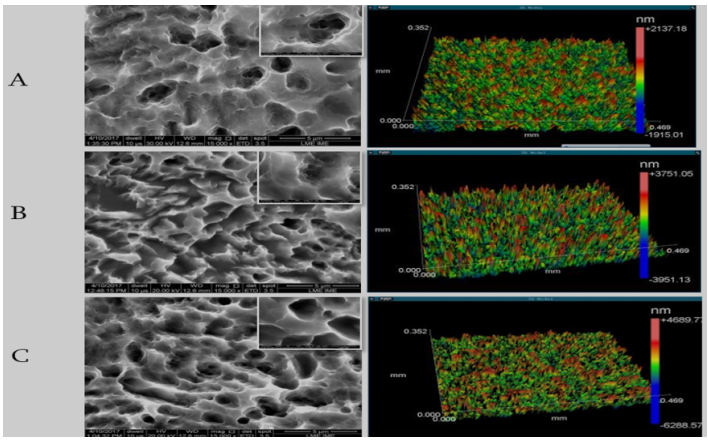


Figure 1: Scanning Electron Microscopy and Interferometry. A: Machined Surface, B: Surface With 20 Minutes Of Acid Treatment, C: Surface With 60 Minutes of Acid Treatment. Original Magnification: 15000X. Detail Inset Magnification 70000X.

Bacterial Adhesion Test

For the present study, standard ATCC (American Type Culture Collection, USA) strains of Streptococcus mutans (ATCC 25175) and Staphylococcus aureus (ATCC 25923) were used, isolated and stored in the Microbiology Laboratory of the São Leopoldo Mandic Dental Research Center (Campinas) , SP. These strains

are kept frozen for subsequent activation in BHI broth culture medium (Brain Heart Infusion, Himedia, India), where they remained for 24 hours in a bacteriological oven at 37 oC, under microaerophilic conditions. After this period, with the aid of a platinum loop, a portion of the culture medium was collected and cultivated in a Petri dish containing BHI agar, so that the strains to be studied could grow, under the conditions described above, in the bacteriological greenhouse. From colonies grown overnight, broths were obtained containing a final density of 15x108 cells/ml, corresponding to factor n. 5 on the McFarland scale (Nefelobac, McFarland Nephelometric Scale, Brazil). To determine bacterial adhesion to different surfaces, bacterial strains were grown on each sample and incubated for 4 hours at 37º C under microaerophilic conditions, as previously described. Samples were gently washed with sterile saline (0.9%) and it was used a BacLight LIVE/DEAD viability kit (Molecular Probe, OR, USA). The kit includes two fluorescent nucleic acids, SYTO9 and propidium iodide. SYTO9 (green fluorescence) identifies viable bacteria, while propidium iodide (Red Fluorescence) identifies non-viable bacteria. To assess viability, 1µL of solution from each area was added to 3mL of sterile saline (0.90%) and then mixed. 70 µL of the solution was dispensed onto each surface and incubated for 15 minutes in the dark at room temperature. Bacterial colonies were examined under a fluorescence microscope (Zeiss, Germany) using a lens (40X). The excitation and emission wavelengths of SYTO9 and propidium iodide were 488 nm and 525 nm, respectively. For each sample, 6 images were taken at standardized positions at each point of the calibration curve. To determine the viability of bacterial species adhered to each type of surface, the area (Arbitrary Units, AU) was measured green zones (viable cells) and red zones (non-viable cells) and the total area of the image (merged), in each surface analyzed for each calibration point of the fluorescence curve, using the ImageJ program (National Institute Of Health, NIH, 167 USA).

The experiments were carried out in triplicate, for each experiment and bacterial species.

Statistical Analysis

To assess viability, 1µL of solution from each area was added to 3mL of sterile saline (0.90%) and then mixed. 70 µL of the solution was dispensed onto each surface and incubated for 15 minutes in the dark at room temperature. Bacterial colonies were examined under a fluorescence microscope (Zeiss, Germany) using a lens (40X). The excitation and emission wavelength of SYTO9 and propidium iodide was 488 nm and 525 nm, respectively. For each sample, 6 images were taken at standardized positions at each point of the calibration curve. To determine the viability of bacterial species attached to each type of surface, the area (Arbitrary Units, AU) of the green zones (viable cells) and red zones (non-viable cells) and the total image area (merged) were measured on each surface analyzed for each calibration point of the fluorescence curve, using the ImageJ program (National Institute of Health, NIH, USA). The experiments were carried out in triplicate, for each experiment and bacterial species. After the data had been assessed for normality and homoscedasticity, comparisons between the three different titanium surfaces in terms of cell count and percentage of area occupied by viable and non-viable S. aureus and S. mutans were performed by average analysis variance for a criterion, followed by Tukey tests. Statistical calculations were performed using a significance level of 5%, using SPSS 23 software (SPSS Inc., Chicago, IL, USA).

Results

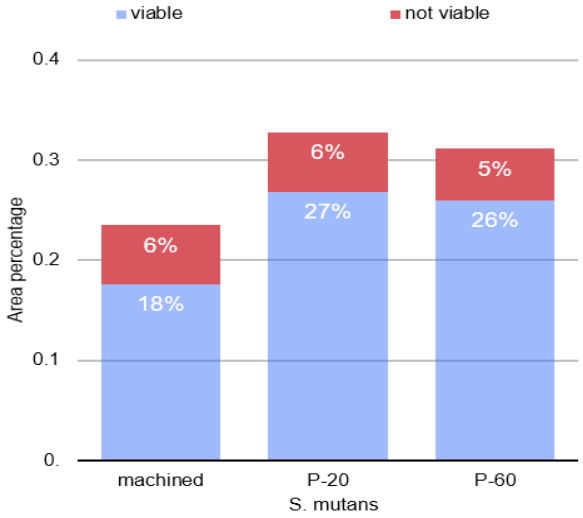
Through one-way analysis of variance, it was found that the count and percentage of area occupied by viable S. aureus ($p<0.001$) and S. mutans ($p=0.042$) were significantly affected by the type of surface. When considering the S. mutans culture, the count and percentage of area were also lower on the machined surface, with no statistically significant difference between the values obtained for the P20 and P60 surfaces (Table 1 and graphs 1 and 3). For S. aureus, the Tukey test identified that the lowest counts and the lowest percentages of area were observed on the machined surface, while the highest values were found on the P20 surface (Table 1 and graphs 2 and 4). The count and area percentage values were intermediate for the P60 surface.

Surface	Viable S. aureus		Viable S. mutans	
	Count (AU)	Area (%)	Count (AU)	Area (%)
Machined	6.148 (1.387) a	9.58 (2.16) a	11.291 (2.478) a	17.60 (3.86) a
P20	20.702 (5.078) c	32.27 (7.91) c	17.216 (3.554) b	26.83 (5.54) b
P60	13.871 (1.571) b	21.62 (2.45) b	16.698 (3.555) b	26.03 (5.54) b
ANOVA	$p < 0.001$	$p < 0.001$	$p = 0.042$	$p = 0.042$

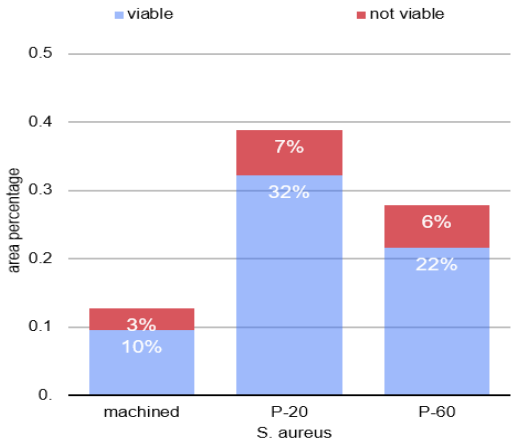
Table 1: Mean values and standard deviations of counts (AU) and percentage of area showing viable S. aureus and S. mutans on different surfaces.

Caption: ANOVA: Analysis of Variance. Averages followed by different letters within the same column differ significantly from each other.

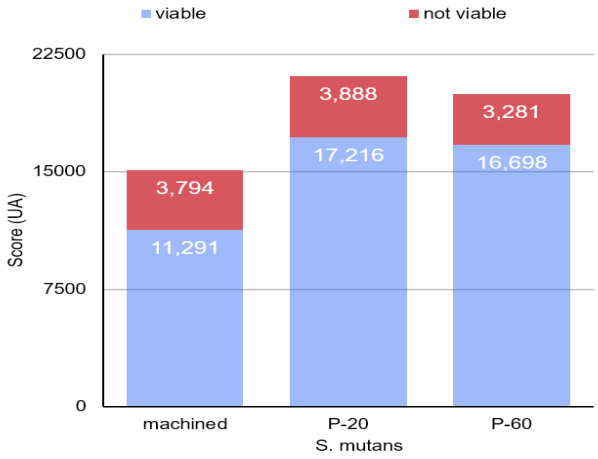
Source: Own authorship.



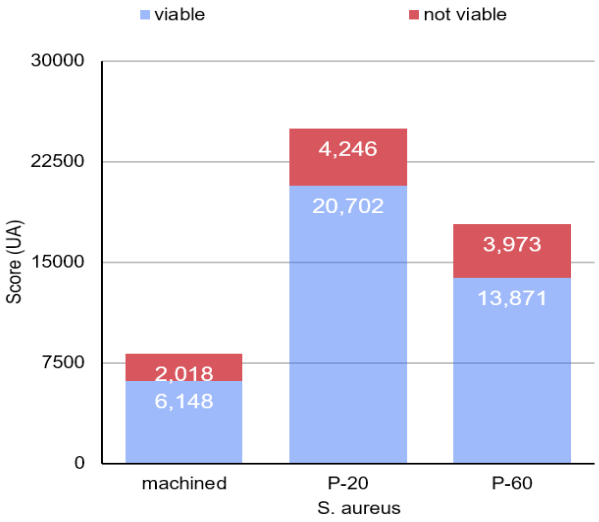
Graph 1: Column diagram of the percentage of area in which viable and non-viable S. mutans were attached to different surfaces.



Graph 2: Column diagram of the percentage of area in which viable and non-viable S. aureus were attached to different surfaces.



Graph 3: Column diagram of the count of viable and non-viable S. mutans on different surfaces.



Graph 4: Column diagram of viable and non-viable S. aureus counts on different surfaces.

Regarding the count and percentage of area data presenting S. aureus (p = 0.573) and S. mutans (p = 0.872), the unfeasible analysis of variance in one criterion demonstrated that there was no statistically significant difference between the surfaces (table 2 and graphs 2 and 4).

Superfície	Non-viable S. aureus		Non-viable S. mutans	
	Count (AU)	Area (%)	Count (AU)	Area (%)
Machined	2.018 (998) a	3.15 (1.56) a	3.794 (1.568) a	5.91 (2.45) a
P20	4.246 (3.996) a	6.62 (6.23) a	3.888 (1.916) a	6.06 (2.99) a
P60	3.973 (2.657) a	6.19 (4.14) a	3.281 (2.202) a	5.11 (3.43) a
ANOVA	p = 0.573	p = 0.573	p = 0,872	p = 0.872

Table 2: Mean values and standard deviations of counts (AU) and percentage of area showing non-viable S. aureus and S. mutans on different surfaces.

Surface

Caption: ANOVA: Analysis of Variance. Averages followed by the same letters within the same column do not differ significantly from each other.

Discussion

Changes in the relationships between the diameters of the implant platforms and the prosthetic components known as the swift platform allowed the maintenance of peri-implant hard tissues at the level of the implant platforms and even above them. This effect caused the industry to change the structure of the cervical region of implants, both in macrogeometric with specific thread designs and in microgeometric with treatment up to the implant platform. The presence of roughness in this region, contrary to what was expected, did not demonstrate a greater presence of bacteria and an increase in peri-implant diseases. The literature shows a lack of conclusive studies on this subject, and more work is needed to achieve an ideal surface for prosthetic components on implants, promoting the health of the peri-implant connective tissue based on tissue adhesion and, consequently, a better seal between the tissue soft and the prosthetic component [6]. The influence of microroughness on cellular behavior around implants is well defined in the literature. Some of these studies have demonstrated that lower roughnesses of less than 0.7um allow fibroblasts to adhere, suggesting that they would alter the behavior in the region of the prosthetic components, and could improve sealing in the peri-implant groove through the formation of collagen fibers perpendicular to the components. Controlled changes in the roughness of the components allow a change in the orientation of the gingival fibers and adhesion to the component. Although these changes will influence the healing of the peri-implant gum, many doubts are still present, whether regarding the type of treatment, the ideal roughness and the bacterial behavior and induction of peri-implant disease [29,30]. In vitro studies have demonstrated that this fact may be possible and that roughnesses close to 0.2 um would have a satisfactory performance [6,30,28]. On the other hand, moderate surface roughness, while favoring the osseointegration of implants, can also provide greater bacterial colonization and, consequently, peri-implantitis [31]. In fact, studies demonstrate that machined surfaces promote less bacterial colonization [32,24,33]. In this study, for S. aureus there was a difference between the groups, while for S. mutans there was no difference between the surfaces when analyzed as viable cells. However, in the evaluation of non-viable cells, there was no difference between the surfaces, including in relation to the machined surface, for both bacteria. When comparing the P-20 and P-60 surfaces, the surface with the lowest roughness showed greater bacterial colonization in the analysis of viable cells for both S. mutans and S. aureus, similar to the results obtained [19]. This result also follows the behavior of fibroblast cells that demonstrate greater adhesion in this type of roughness. In a human study, it was concluded that the laser-treated surface is promising in positively influencing peri-implant connective tissue wound healing. The results demonstrated that the topographic nature of healing pillars can positively influence mucosal healing and molecular expression [25,27,34,22,44]. Rough surfaces show greater adhesion of certain bacterial species only in the initial moments. At more advanced stages, smooth and rough surfaces behave similarly in terms of the number of colonies present in the biofilm [35-38,17]. Individual and systemic characteristics inherent to each individual also seem to have a decisive effect on bacterial colonization, even having a greater impact than the material of the prosthetic abutments itself [39,29]. A study in dogs demonstrated that implants maintained suprabony with rough cervical treatment and without surface treatment demonstrated better quality of peri-implant tissues, including vertical bone increase, without showing the presence of disease in any of the cases [40,41]. In another study in dogs, the results suggest that the healing of hard and soft tissues around implants and abutments is similar when comparing sandblasted surfaces with surfaces machined and turned with nanotubes. Both resulted in similar soft tissue contact values as well as connective tissue fiber orientation [26]. As there is no direct competition between bacteria and fibroblast cells, despite the study showing greater adhesion of bacteria in the samples, it cannot be concluded that the roughness of the prosthetic components would actually increase the risk of infectious inflammatory peri-implant disease [42-44].

Conclusion

Despite the limitations of the study, it is possible to conclude that increasing the roughness of titanium discs treated with acid etching at different times increases bacterial adhesion of S aureus and S mutans in vitro.

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Authors' contributions

FTB and EFM interpreted and analyzed the data collected, contributed to the drafting of the paper and revised it critically, and were major contributors in writing the manuscript. RM, VZS, and CNE contributed to the concept/design of the study and the final manuscript. VZS and RM critically revised and contributed to the final manuscript. All authors read and approved the final version to be published.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This work has received exemption from the Research Ethics Committee for Human Beings of the São Leopoldo Mandic Institute and Research Center, Campinas/SP as it is exclusively laboratory research, without the involvement of human beings or materials (Protocol 2016/06460, annex 1).

Consent for publication

Not applicable.

Competing interests

Flavia Tatiane Barbosa Lima[a];Vilton Zimmermann de Souza[a], Rafael Manfro[a], Carlos Nelson Elias[c], Elizabeth Ferreira Martinez[b*] state that they have no conflicts of interest.

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