Research, Society and Development, v. 11, n. 16, e462111638288, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i16.38288

Adherence of *Escherichia coli* and blood elements in titanium discs submitted to anodic oxidation. In vitro study

Aderência de Escherichia coli e elementos sanguíneos em discos de titânio submetidos à oxidação anódica. Estudo in vitro

Adherencia de Escherichia coli y elementos sanguíneos en discos de titanio sometidos a oxidación anódica. Estudio in vitro

Received: 11/21/2022 | Revised: 12/04/2022 | Accepted: 12/06/2022 | Published: 12/15/2022

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Abstract
Modifications of implants by means of surface treatments are performed to optimize the biochemical interactions of the bone deposition process. However, if on the one hand they favor the adhesion of blood elements, on the other hand they can also enable the formation of biofilm. This study evaluated the adherence of blood cells and *Escherichia coli* in titanium discs submitted to surface treatments by anodic oxidation (OA), sandblasting followed by acid etching (JAT) compared with untreated discs (Li). To evaluate the adherence of microorganisms were performed: atomic force microscopy; Field Emission Gun Scanning Electron Microscopy (FEG-SEM); biofilm formation was evaluated by spectrophotometer turbidity analysis and colony forming unit (CFU/mL) before and after simulated brushing. For blood cell adherence, the blood collected from a patient was deposited and fixed on the discs and analyzed in the FEG-SEM being classified according to the "Blood Element Adherence Index". The results showed a slight increase in the adhesion of microorganisms in samples treated by anodic oxidation. However, the microorganisms were distributed singly and not in conglomerates, with no biofilm formation unlike the Li group. Regarding the adherence of blood elements, the Li group showed higher adherence and lower amount was found in the JAT group, but there was no statistically significant difference between the groups. The results of this study suggest that anodic oxidation treatment may favor the adherence of blood cells and fibrin mesh, contributing to the early stages of bone deposition.
Keywords: Titanium; Blood cells; Cell adhesion; Bacterial adhesion; Peri-implantitis.

Resumo
Modificações de implantes através de tratamentos de superfície são realizadas para otimizar as interações bioquímicas do processo de deposição óssea. Mas se por um lado favorecem a adesão de elementos sanguíneos, também podem permitir a formação de biofilme. Este estudo avaliou a aderência de células sanguíneas e Escherichia coli em discos de titânio submetidos a tratamentos de superfície por oxidação anódica (OA), jateamento e ataque ácido (JAT) em comparação com discos sem tratamento (Li). Para avaliar a aderência de microorganismos foram realizados:

microscopia de força atômica; microscopia eletrônica de varredura por emissão de campo (FEG-SEM); a formação de biofilme foi avaliada por análise de turbidez por espectrofotômetro e unidade formadora de colônia antes e depois da escovação simulada. Para a aderência de células sanguíneas, o sangue coletado de um paciente foi depositado e fixado nos discos e analisado no FEG-SEM sendo classificado de acordo com o "Índice de aderência de elementos sanguíneos". Os resultados mostraram um leve aumento na adesão de microorganismos em amostras tratadas por oxidação anódica. No entanto, os microorganismos foram distribuídos isoladamente e não em conglomerados, sem formação de biofilme diferente do grupo Li. Com relação à aderência dos elementos sanguíneos, o grupo Li apresentou maior aderência e menor quantidade foi encontrada no grupo JAT, mas não houve diferença estatisticamente significativa entre os grupos. Os resultados deste estudo sugerem que o tratamento de oxidação anódica pode favorecer a aderência das células sanguíneas e da rede de fibrina, contribuindo para os estágios iniciais da deposição óssea.
Palavras-chave: Titânio; Células sanguíneas; Adesão de células; Adesão bacteriana; Peri-Implantite.

Resumen

Se realizan modificaciones de los implantes mediante tratamientos de superficie para optimizar las interacciones bioquímicas del proceso de deposición ósea. Pero si por un lado favorecen la adhesión de elementos sanguíneos, también pueden permitir la formación de biofilm. Este estudio evaluó la adhesión de células sanguíneas y Escherichia coli en discos de titanio sometidos a tratamientos superficiales por oxidación anódica (OA), chorreado seguido de grabado ácido (JAT) en comparación con discos no tratados (Li). Para evaluar la adherencia de los microorganismos, se utilizó la microscopía de fuerza atómica, la microscopía electrónica de barrido de emisión de campo (FEG-SEM) y se evaluó la formación de biopelículas mediante el análisis de turbidez del espectrofotómetro y las unidades formadoras de colonias antes y después del cepillado simulado. Para la adherencia de las células sanguíneas, la sangre recogida de un paciente se depositó y se fijó en los discos y se analizó en el FEG-SEM y se clasificó según el "índice de adherencia de los elementos sanguíneos". Los resultados mostraron un ligero aumento de la adhesión de los microorganismos en las muestras tratadas por oxidación anódica. Sin embargo, los microorganismos se distribuían aisladamente y no en conglomerados, sin formación de biopelículas, a diferencia del grupo Li. En cuanto a la adherencia de los elementos sanguíneos, el grupo Li mostró mayor adherencia y se encontró menor cantidad en el grupo JAT, pero no hubo diferencias estadísticamente significativas entre los grupos. Los resultados de este estudio sugieren que el tratamiento de oxidación anódica puede favorecer la adherencia de las células sanguíneas y la red de fibrina, contribuyendo a las etapas iniciales de la deposición ósea.
Palabras clave: Titanio; Células sanguíneas; Adhesión celular; Adhesión bacteriana; Periimplantitis.

1. Introduction

With the advancement of implant dentistry, dental rehabilitation through dental implants has increased. Similarly, surface modifications that seek to optimize the repair, allowing less time for healing, have been relevant in recent dental studies aimed increasingly at implementing early loading. Several studies have been conducted on the influence of surface modifications osseointegration (Albrektsson et al., 1981; Smeets et al., 2016). Thus, the physical-chemical properties of implants such as wettability, contact angle, roughness, chemical composition are some characteristics that have been modified through surface treatments such as laser irradiation (Hallgren et al., 2003), acid treatment and alkaline etching (Matos et al., 2022; Oliveira et al., 2014), anodization (Le Guehennec et al., 2007) in order to optimize osseointegration.

A proposal under discussion refers to surface treatment by anodic oxidation. The anodic oxidation treatment is obtained through the electrochemical process, producing a nanotopographic titanium dioxide layer and stands out among the proposals for its surface without acute characteristics and with good capacity to retain liquids and bone tissue (Hall & Lausmaa, 2000). Besides being resistant to wear due to its high adherence to the implant, allowing it to be installed without loss of particles (Al-Nawas et al., 2008).

Although there has been a relative abundance of research on surface treatments, the initial healing process is not addressed in studies evaluating implant surfaces or titanium discs. The adhesion of blood cells is the first step in the healing process and it is of great importance for the subsequent processes that are responsible for the success of a dental implant.

On the other hand, while surface modifications have been reported as important for the osseointegration process, in some cases they can also facilitate microbial adhesion, mainly due to the increased surface roughness (Al-Ahmad et al., 2013; Burgers et al., 2010). An implant surface should minimize bacterial adhesion, but be sufficiently able to allow biochemical

and cellular interactions for osseointegration (Grossner-Schreiber et al., 2001). Similarly to natural teeth, dental implants are colonized by the bacteria of dental biofilm (Aoki et al., 2012; Mombelli, 1993) and the development of inflammatory lesions such as mucositis and perimplantitis, for example, can be established if biofilm is perpetuated (Jepsen et al., 2015).

Lack of oral hygiene, history of periodontitis or smokers are indicators of periimplantitis risks and may lead to loss of the implanted element (Ephros et al., 2020). E. coli has been reported in studies for experimental periodontitis, showing rapid and aggressive bone destruction (Gurkan et al., 2009) and is increasingly associated with peri-implantitis (Wang et al., 2020). Its use to evaluate bacterial adhesion on dental implant surfaces is found in the literature (Ferreira, Babu, Tipton, & Hottel, 2015). Brushing is well established as an effective method for the control of biofilm for the prevention of periodontal and periimplant diseases (Jepsen et al., 2015), however, it may be modifying factor of the implant surface (Fais et al., 2012; Kim et al., 2019). Based on these data, studies evaluating the adhesion of microorganisms on titanium implant surfaces, as well as the influence of the brushing procedure on the surface modification and its impact on biofilm formation are needed.

Therefore, this study compared the adhesion of blood cells and biofilm formation in titanium discs submitted to anodic oxidation before and after the simulated brushing procedure and analyzed the possible influence of the simulated brushing procedure on the surface roughness of titanium discs.

2. Methodology

This study was approved by the Research Ethics Committee of the State University of Ponta Grossa, Paraná - Brazil number 1,076,908.

Sample preparation

Commercially pure titanium discs (diameter 6 mm; thickness 2 mm - Neodent®, Curitiba, Brazil) were used in this study. Three groups were obtained (n=20 per group) according to their surface treatment: control group without treatment (Li), sandblasting followed by acid etching (Jat) and titanium discs submitted to anodic oxidation (OA). The Li and OA groups were mechanically polished with 600 and 1200 grit SiC and ultrasonically washed with distilled water, ethyl alcohol and acetone for 10 minutes each solution. The anodic oxidation was performed through a system that consists of a programmable DC 600 V 8 A source, microcomputer for control, oscilloscope and electrolytic tank. Using current density 90 mA/cm²/100s and variable voltage (galvanostatic mode) and electrolytes: calcium acetate monohydrate 0.14 mol/L, sodium biphosphate monohydrate 0.06mol/L.

Sample Decontamination

The samples were sequentially immersed in 70° alcohol for 10 minutes, hypochlorite 1% for 3 minutes, alcohol 70° for 10 minutes. They were washed with sterile physiological solution for 1 minute, always in constant manual agitation. After drying, the specimens were sterilized by ultraviolet light for 60 minutes (each side) (Ma et al., 2014).

Adherence of blood cells

A total of 10 mL of blood was collected from one of the researchers by puncturing the peripheral blood to obtain the blood cells. The blood was taken and the coagulation tests were performed at the School Laboratory of the University. The blood was deposited on the discs (1 drop per sample) and kept for 20 minutes in a humidifying chamber. The following processes were as described by Tsurumaki JN et al., 2013(Tsurumaki et al., 2013): The specimens were washed 3 times for 5 minutes with phosphate buffer solution (PBS, pH 7.0) by means of agitator. The samples were identified and fixed in 1% formaldehyde solution in PBS for 15 minutes. After 3 washes of 5 minutes in PBS, they were incubated for 10 minutes in

0.02M glycine in PBS and washed again. They were fixed in 2.5% glutaraldehyde in PBS for 30 minutes and washed. The samples were dehydrated in ethanol series: 25%, 50%, 75%, 95% and 100% for 10 minutes in each solution. After drying, by means of a CO_2 critical point machine they were kept in a vacuum desiccator for at least 24 hours (Tsurumaki et al., 2013). The discs were submitted to FEG-SEM (MIRA 3 LMH, TESCAN) analysis to obtain photomicrographs.

The images were analyzed by a calibrated, experienced and "blind" examiner for specimen identification, and classified according to the "Blood Element Adhesion Index" (Theodoro et al., 2006): (0) absence of fibrin network and blood cells; (1) scarce fibrin network and/or blood cells; (2) moderate fibrin network and a moderate number of blood cells; 3) a dense fibrin network and trapped blood cells.

Biofilm formation

The specimens were aseptically placed in test tubes containing 5 mL of brain heart infusion (BHI) broth, and 1 mL of *Escherichia coli* suspension (ATCC 25922) at a concentration of 108 cells/mL was added to the tubes. This suspension was obtained from *E. coli* colonies grown in Petri dishes containing BHI culture medium, diluted in sterile saline solution in a test tube and vortexed until a turbidity equivalent to the number one tube on the Mac Farland 0.5 scale was obtained and proved by spectrophotometric analysis. Tubes containing *E. coli* were taken to a bacteriological oven at 37°C/24h to promote the adherence of microorganisms to titanium discs. In order to verify the adherence of *E. coli* to the specimens, the tubes containing the material detached from the tested materials were read in the spectrophotometer. The quantification of the microorganisms adhered to the materials was also performed by dilution of the suspension obtained and aliquots of 0.1 mL of the pure suspension and each dilution (10-1 and 10-2) were seeded in duplicates in Petri plates containing BHI culture medium (Brain Heart Infusion Agar, BHI, Difco) and then taken to a bacteriological oven (Park et al., 2012).The CFU/mL were counted by means of a colony counter on the plates.

Field Emission Gun Scanning Electron Microscopy (FEG-SEM)

Regarding the adherence of blood cells, the specimens were washed with 0.1 mol/L sodium cacodylate buffer at pH 7.4. The specimens were fixed in 0.1 mol/L solution containing 2% paraformaldehyde and 2.5% glutaraldehyde for 1 hour and dehydrated in increasing order of graduated ethanol. The samples received gold deposition (SC7620 – Quorum, ENGLAND) and analyzed in FEG-SEM (MIRA 3 LMH, TESCAN).

For *E. coli* adherence, the specimens were fixed for 4 hours with 2.5% glutaraldehyde then washed 4 times with PBS for 1 minute, distilled water 2 times for 1 minute, dehydrated with ethanol at 60, 70, 80 and 90% and finally 3 times with 100% each for 10 minutes. The samples were dried on silica for 48 hours and received gold deposition (SC7620 - Quorum, ENGLAND) to be examined by FEG-SEM (MIRA 3 LMH, TESCAN) (Castillo et al., 2005).

Atomic force microscopy

For surface roughness evaluation, the specimens submitted to *E.coli* adhesion were scanned by an atomic force microscope (SHIMADZU SPM 9600) in 3 different areas to obtain 15 measures of roughness for each test group (Giessibl, 2003). The results were expressed in mean roughness (Ra).

Microhardness

A Vickers tip, with a force of 200 gf, was used against the surface of the titanium material for 15 seconds in samples submitted to *E.coli* adhesion.

Simulated brushing

The specimens were submitted to brushing by means of a brushing machine (MSEt - 1500W power and voltage of 220 Vac-60 Hz + earth, weight approximately 70Kg), at 20,000 movements (Tanoue, Matsumura, & Atsuta, 2000), which correspond to two years of brushing, with a load of 400gf (Kawai, Iwami, & Ebisu, 1998). The brushes used were nylon bristles (head 25 and 32 mm by 8 and 11 mm wide; 14 tufts: 3x6.3x8 rows; soft nylon artificial bristles with rounded tips). The specimens were brushed with Colgate®MFP toothpaste classified as medium abrasive and of great national consumption (Andrade Junior et al., 1998).

Statistical analysis

The statistical test used for the blood element adhesion index was the Kruskal-Wallis non-parametric test and in case of a value of $p \leq 0,05$ the comparison between the midpoints of the samples would be performed using the Mann-Whitney test. *E. coli* adhesion, mean roughness and microhardness before and after simulated brushing of the different surface treatments tested were submitted to ANOVA one-way and Tukey's post-test. The minimum statistical significance was established in $p \leq 0.05$.

3. Results

All groups of tested implant surfaces showed adhesion of blood elements. Averages: Li= 2.20; OA= 1.90 and JAT= 1.70. There was no statistically significant difference between the materials in the Blood Element Adhesion Index (considering $p<0.05$), the means between the implant surfaces were very similar. The proportions of samples per score are represented in Table 1 showing the highest frequency of the groups present in score 2.

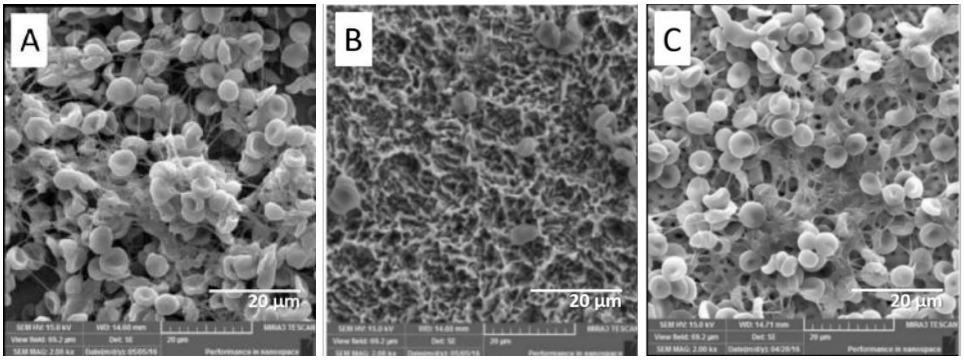
Table 1. Frequency of the Index of Adhesion of Blood Elements in the studied groups (n=10 for each group).

Score	Li	Jat	OA
0	0	2	0
1	0	1	1
2	8	5	9
3	2	2	0

Source: Authors.

The images obtained by FEG-SEM (Figure 1) demonstrate greater adhesion of blood cells in the OA and control groups compared to the JAT group.

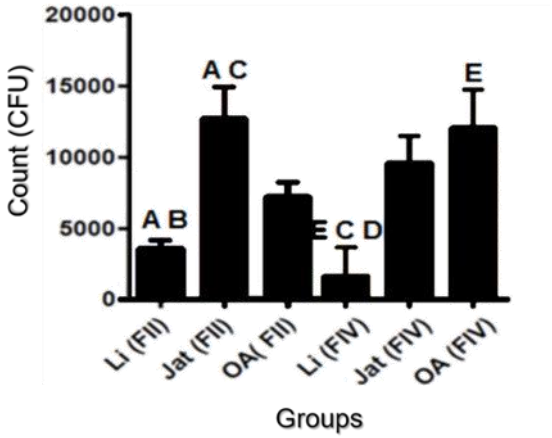
Figure 1 - Electronic Scanning Microscopy with Field Emission Source (FEG-SEM) of Blood Element Adhesion on tested implant surfaces A - Li; B - Jat. C - OA. Magnification x2000.



Source: Authors.

Microbial adhesion experiments followed according to the phases: FI=Phase before brushing and before biofilm formation; FII=Phase before brushing and after biofilm formation; FIII= Phase after brushing; FIV=Phase after brushing and after biofilm formation. The growth and adherence of the biofilm to the specimens before and after brushing are represented in Figure 2. The results show significant statistical difference between the groups in the different stages.

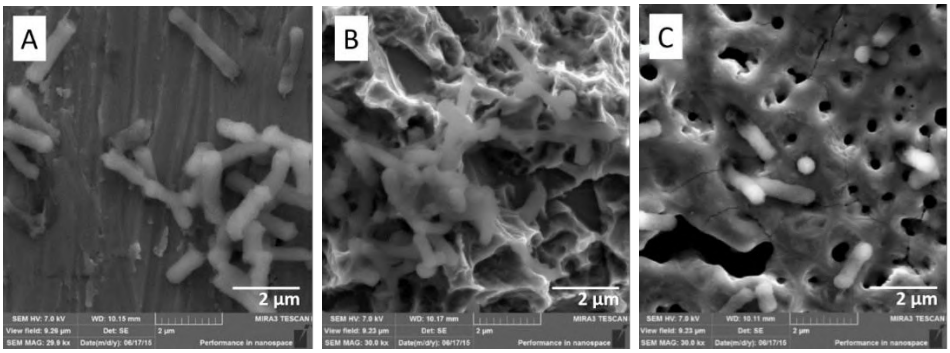
Figure 2 - *E. coli* adherence before and after simulated brushing of the different surface treatments. Significant differences (p=0.0004) were indicated with letters (A, B, C, D, E): between group Li (FII) and group Jat (FII); group Li (FIV) and group Jat (FII); group Li (FIV) and group OA (FIV).



Source: Authors.

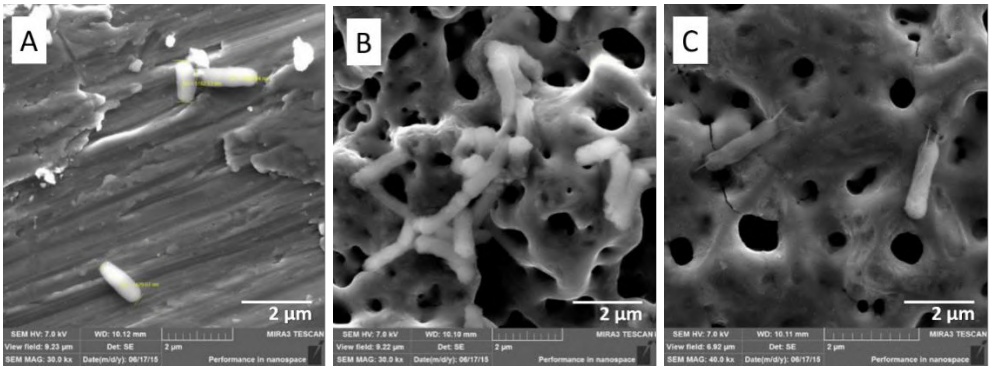
In the images by FEG-SEM (Figure 3 and Figure 4) it is possible to observe that even after the simulated brushing there is still microbial adhesion in all groups.

Figure 3 - Images obtained by Field Emission Gun Scanning Electron Microscope (FEG-SEM). Specimens with biofilm formation before the simulated brushing (Phase II). (A) Li. (B) Jat. (C) OA. Magnification x30000.



Source: Authors.

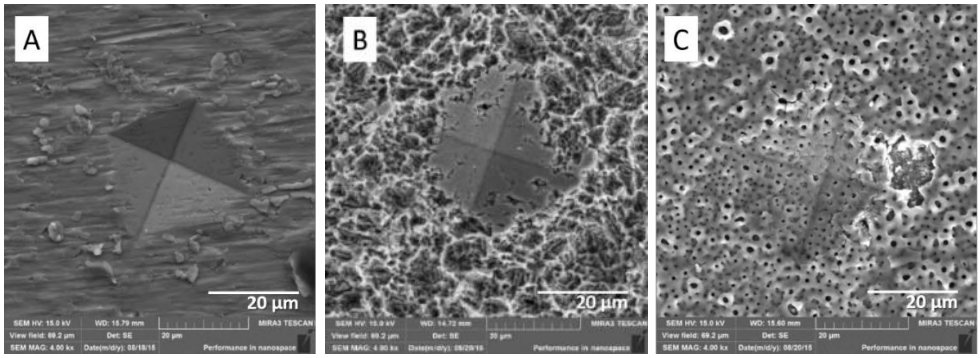
Figure 4 - Images obtained by Field Emission Gun Scanning Electron Microscope (FEG-SEM). Specimens with microbial adhesion after simulated brushing (Phase IV). (A) Li. (B) Jat. (C) OA. Magnification x30000.



Source: Authors.

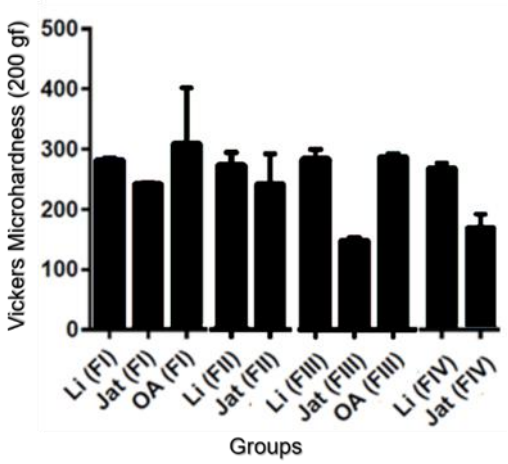
The results of the microhardness test (Figure 5 and Figure 6) in the different phases showed that there was no significant difference (p =0,0924) between the same groups before and after brushing and between groups before brushing and after brushing.

Figure 5 - Images by Field Emission Gun Scanning Electron Microscope (FEG-SEM) for the Vickers microhardness test of 200 gf for 15 seconds in the different surface treatments, phase I. (A) Li. (B) Jat. (C) OA. This test was also performed in phase II, phase III and phase IV. Magnification x4000.



Source: Authors.

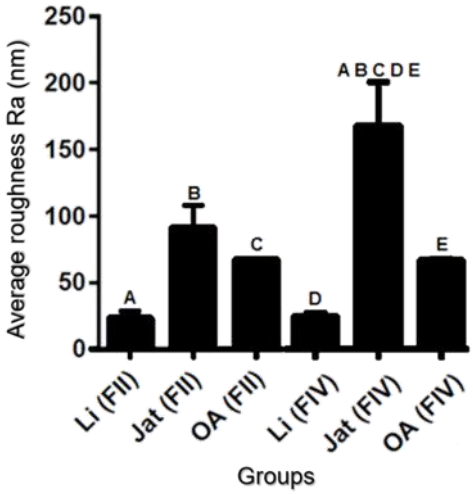
Figure 6 - Microhardness of the surface treatments tested in the reference phases (I and III) and the microbial adhesion phases before and after simulated brushing (II and IV). Statistical analysis: ANOVA of one factor and Tukey's post-test ($p < 0.05$) resulting in $p = 0.0924$, there was no significant difference between the same groups before and after brushing and between groups before and after brushing.



Source: Authors.

The roughness before and after brushing are shown in Figure 7. The Jat group demonstrated significant statistical difference between the other groups and among themselves in both phases (II and IV).

Figure 7 - Roughness of surfaces in the microbial adhesion phases before and after the simulated brushing for the different surface treatments tested. Significant differences ($p < 0.0001$) were indicated with letters (A, B, C, D, E): between groups Li, Jat, OA, da (FII) and Jat group (FIV); groups Li, OA, (FIV) and Jat group (FIV).



Source: Authors.

4. Discussion

The physicochemical characteristics of surfaces, such as surface roughness, material microhardness, corrosion and surface free energy influence the osseointegration process of dental implants (Rupp et al., 2014; Smeets et al., 2016). However,

studies have shown that certain conditions such as exposure of the metal to pH, humidity and oral hygiene using toothpaste can contribute to surface alteration and consequently interfere with the physicochemical properties (Lindholm-Sethson & Ardlin, 2008). In vitro studies have shown corrosion of commercially pure titanium due to prophylactic agents containing fluoride (Huang, 2002; Lindholm-Sethson & Ardlin, 2008; Stajer et al., 2008). However, the results of microhardness presented in this study show that no significant modification in the surface of the titanium discs was detected, regardless of the type of treatment that was performed.

In addition, high fluoride concentrations and low pH have been reported for raising the surface roughness and degradation of titanium (Fais et al., 2012). In this study an opposite result was observed for most groups, that is, a decrease of roughness after tooth brushing was found. Although roughness is described by some authors as influencing initial bacterial adhesion (Burgers et al., 2010; Han et al., 2016), no directly proportional correlation between titanium surface roughness and bacterial adhesion count was observed. However, lower adherence of bacteria was demonstrated in the phase after brushing in most groups, emphasizing the importance of oral hygiene by the patient for the control of dental biofilm.

Biofilm formation is considered necessary for expression of pathogenicity and development of periimplantitis, thus, bacteria when isolated may not present pathogenic effects (Bernimoulin, 2003). The results showed that discs treated by anodic oxidation obtained an increase in bacterial adhesion compared to the Li group. However, differently from the Li group, it is possible to observe from the FEG-SEM figures that in the anodic oxidation treatment the bacteria were isolated and not in conglomerates. It was observed that the discs treated by anodic oxidation had an increase in roughness, and there was an increase in bacterial adhesion only after brushing, with a significant difference with the Li group (IVF). However, the roughness of the discs treated by anodic oxidation before and after brushing showed no variability. The Jat group showed higher roughness after brushing, but no significant difference was observed in the different phases with the different groups in the adherence of bacteria.

This research has shown that surface roughness may be related to the quantitative increase of *E. coli* colonization. However, smooth surfaces can also show adhesion of microorganisms and higher biofilm formation. Research has proven that bacteria when in communities can have an exacerbation of their deleterious effects to our body and oral cavity (Dhotre et al., 2017; Furst et al., 2007; Koka et al., 1993). Therefore, surface treatment may not be the main factor related to the presence of periimplant diseases. It should be taken into account that oral conditions in vivo, saliva, feeding, systemic conditions of the patient, are almost impossible to reproduce in vitro, this could explain the differences in biofilm formation in in vivo and in vitro studies.

Pinto et al 2013 (Pinto et al., 2013) evaluated the adherence of blood elements on exposed dental surfaces to the main toxic substances of cigarette, nicotine and cotinine, associated with non-surgical periodontal treatment in vitro. Observing a greater adherence when scaling and root planing were performed. However, the adherence of these elements considering the surface treatment aspects in implants were not addressed in previous studies. Considering the different types of treatment in titanium discs and their roughness characteristics, in this study no statistically significant differences were observed between the groups. The highest adherence was found in the Li group and the lowest in the Jat group. For a better understanding of these results, other physical-chemical characteristics of the surfaces, such as surface free energy, wettability (Rupp et al., 2006) that were not contemplated in this study need to be analyzed. Furthermore, although greater adhesion has been observed on Li surfaces, studies have already well consolidated the relationship between osseointegration and surface roughness (Le Guehennec et al., 2007). The initial step may be greater on smooth surfaces, but during the process, the rough surface favors greater bone deposition at the bone-implant interface (Buser et al., 1991; Suzuki et al., 1997).

5. Conclusion

All surfaces allowed the adhesion of blood cells and fibrin mesh. A slight increase in the adherence of microorganisms was found in the samples treated by anodic oxidation, however, there was no statistically significant difference when compared to specimens from the same group that were subjected to simulated brushing. The increase in surface roughness may explain the major accumulation of microorganisms. So, it was concluded that the surface treatment in titanium discs can lead a slight increase in the accumulation of microorganisms, however, we still observed a large biofilm formation on smooth discs.

Acknowledgments

We thank Neodent, Curitiba-Paraná, for supplying the titanium discs.

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) and Fundação Araucária (FAPPR).

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