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(Organizadora)

Comunicação Científica e Técnica em Odontologia

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APRESENTAÇÃO

A Odontologia vem ampliando cada vez mais sua área de atuação dentro do campo da saúde. Hoje aliamos o conhecimento teórico de base às novas tecnologias e técnicas desenvolvidas através de pesquisas para elevar a qualidade e atingir excelência na profissão.

Diante da necessidade de atualização frequente e acesso à informação de qualidade, este E-book, composto por dois volumes, traz conteúdo consistente favorecendo a Comunicação Científica e Técnica em Odontologia.

O compilado de artigos aqui apresentados são de alta relevância para a comunidade científica. Foram desenvolvidos por pesquisadores de várias instituições de peso de nosso país e contemplam as mais variadas áreas, como cirurgia, periodontia, estomatologia, odontologia hospitalar, bem como saúde do trabalhador da Odontologia e também da área da tecnologia e plataformas digitais.

Espero que possam extrair destas páginas conhecimento para reforçar a construção de suas carreiras.

Ótima leitura!

Profª. MSc. Emanuela Carla dos Santos

SUMÁRIO

CAPÍTULO 24

CORROSION RESISTANCE AND ANTI-BIOFILM EFFECT OF ROCK ROSE REMEDY: A POTENTIAL PREVENTIVE MEASURE IN IMPLANT THERAPY

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ABSTRACT: Staphylococci is the leading etiologic agent of implant-related infection. In presence of rock rose (Helianthemum nummularium) floral remedy, we evaluated the corrosion behavior of commercially pure titanium as well as quantified the Staphylococcus aureus biofilm formation. To analyze the corrosion resistance of pure titanium, electrochemical corrosion tests were performed. We submitted pure titanium samples into a physiological (pH 6.50) or pathogenic (pH 2.50) artificial saliva environment at 37oC. For the in vitro static biofilm assays, a multivirulent Staphylococcus aureus strain was used for assessment of biofilm formation over pure titanium disks in the absence/presence of rock rose floral. Triplicates were performed for both corrosion tests and biofilm experiments. P values were determined by two-way analysis of variance for pairwise comparisons (corrosion) and Student's t-test (biofilm assays). Results were considered significant when p-value < 0.05. Rock rose floral treatment reduced Staphylococcus aureus biofilm formation on titanium surface and promoted a higher titanium corrosion resistance in artificial saliva at low pH. We have shown that rock rose remedy can prevent bacteria adhesion over the pure titanium in a healthy oral environment (pH 6.50) and did not interfere in the titanium corrosion response.

KEYWORDS: *Corrosion; Titanium; Staphylococcus aureus; Peri-Implantitis; Rock Rose; Floral*

INTRODUCTION

Community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA), has been associated with severe infections and high mortality rates. The pathogenesis of pneumonia [1], endocarditis [2], catheter and skin [3] infection caused by USA300 clone have been extensive researched. On an oral environment, both Staphylococcus aureus and Staphylococcus epidermidis were able to grow as part of the subgingival biofilm on hydroxyapatite disks and on titanium surfaces. Although Staphylococcus aureus is a non-oral bacteria, when integrated to the oral microflora leads to either aggressive periodontitis or peri-implantitis [4,5].

Peri-implantitis is an infectious disease that occurs in the tissue surrounding a dental implant with loss of supporting bone resembling periodontitis on natural teeth [6,7]. Prognosis of

the affected implant will be contingent upon early detection and treatment [8]. Peri-implantitis therapies depend on the amount of bone loss and on the esthetic impact of the implant, comprising a nonsurgical or a surgi- cal phase. The former includes debridement by mechanical means, ultrasonic, or laser devices, either alone or combined with antiseptic and/or antibiotic agents. For the surgical phase regenerative techniques are usual approaches [9]. In this light, implantoplasty, chemical decontamination or debridement could modify the titanium implant surface favoring bacteria biofilm accumulation. Excessive mechani- cal stress, poor design of the implant and titanium corrosion are also important factors in the onset and development of peri-implantitis. [10-13].

Titanium and its alloys have been widely used either for orthopedic replacements or dental implants [14] because of their low specific mass, good mechanical properties, high corrosion resistance and adequate biocompatibility [15]. The corrosion resistance of titanium is due to the formation of a stable film of titanium oxide on its surface; however, depending on the conditions of the medium where the tita- nium is exposed, dissolution of this oxide may occur at some points, especially within an acidic environment [16]. Many electrochemical techniques are used to evaluate the stability of this passive film of titanium oxide, such as anodic polarization, impedance spectroscopy, electrochemical noise, etc. These techniques intend to obtain parameters related to the titanium-solution interaction. The presence of ag- gressive agents may lead to the dissolution of the passive film on the titanium surface. For instance, low pH that occurs during infections along with fluoride ions represent harmful conditions to titanium [17-19].

Titanium corrosion and wear processes of dental implants can release ions or debris into the tissue resulting on a hypersensitivity response reported in susceptible patients [20]. The titanium oxide film over part of the surfaces reduces to a very low intensity the trans- formation of metal into ions. This oxide film passivates the surface and also strongly reduces the corrosion [21]. The passivation, however, does not mean immunity against corrosion. Indeed, several situations can increase the instability of the passive film, such as pitting, fret- ting, galvanic effects, and again the low pH [22].

Artificial saliva is a common medium used to simulate the oral environment for corrosion evaluation. The effect of pH as low as 3.0 tends to increase the roughness of pure and Ti-6Al-4V in saliva [23] predisposing to Staphylococcus aureus adhesion. In this sense, the use of adjuvant therapies that could avoid biofilm-related infections are welcome, as long as they do not interfere with the titanium oxide film stability. Hence, the search for treatment that could reduce the problems related to implant failure is desirable.

Floral therapies are recognized by the World Health Organization - WHO as an alternative treatment. Furthermore, knowing that the Brazilian experience in medicine and dentistry has shown that these therapies do not produce side effects, as well as being available at low cost to patients [24], we hypothesized that rock rose floral therapy could intervene with bacteria adhesion without jeopardizing the titanium corrosion

properties especially in low pH medium. The performed assays were intended to verify this hypothesis.

MATERIALS AND METHODS

In vitro static biofilm assays

For in vitro static biofilm assays, the overnight culture of a multivirulent methicillin resistant wild-type strain Staphylococcus aureus (USA300) were diluted 1:100 in TSB supplemented with 0.5% glucose (TSB-G). Diluted bacteria were mixed with 20% pooled human plasma and used for assessment of biofilm formation on commercially pure titanium (Ti-CP) samples that were fixed to the bottom of a 12-well polystyrene plate with Lubriseal grease (Thomas Scientific) and sterilized by ultraviolet irradiation. Multi-well plates were incu- bated at 37°C with shaking at 100 rpm for one hour and then further incubated at 37°C without shaking for 24h. The wells were washed three times with phosphate buffered saline to remove non-adherent cells. Adherent biofilms were fixed with methanol, stained with crys- tal violet and washed three times with sterile water. Biofilm biomass formed on the pure titanium samples were determined by solubiliz- ing crystal violet with 33% acetic acid as previously described elsewhere [25] and measured at 490-nm light wavelength using microtiter plate reader (Biorad). Pre-treated titanium samples were immersed into 2.0 mL of rock rose floral (Helianthemum nummularium from Healing herbs® Bach Flower Essences, Ltd) for 18h before adding Staphylococcus aureus culture. All biofilm biomass experiments were performed in triplicates, and at least three separate experiments were performed with similar results.

Unpaired Student's t-test with the Tukey multiple comparison post-hoc test, was used to assess the statistical significance of between- group differences in bacterial count in vitro biofilm biomass.

Electron microscopy

Fixed titanium samples were processed according to standard methods and sputter coated with gold and analyzed using JEOL JCM- 5000 Neoscope scanning electron microscope.

Corrosion tests

The corrosion test apparatus consisted of a standardized three-electrode cell. A silver-silver chloride electrode (Ag/AgCl at 3M potas- sium chloride) was used as the reference electrode and a platinum foil used as the counter electrode. The working electrode was repre- sented by commercially pure titanium (Ti-CP) samples which were embedded in an autopolymerizing epoxy resin with 1.0 cm2 of exposed area. Prior to each measurement, the sample surface was abraded using a 600 grade emery

paper under water flow, subsequently washed with double-distilled water, degreased with ethanol and dried with warm air. The temperature of the electrochemical cell was maintained at 37.0 ± 0.2°C using a thermostat system. A computer controlled potentiostat (Reference 600 model, Gamry Instruments) was employed to carry on the electrochemical tests.

The artificial saliva solution was used as the electrolyte, respecting the following composition: KCl 960 mg, NaCl 674 mg, MgCl2 41

mg, K2HPO4 274 mg, CaCl2 117 mg, D-sorbitol 24.0g, carboxymethyl cellulose 8.0g, completed with deionized water to a final volume of

L [26]. The pH was adjusted to 6.50 or 2.50 using sufficient lactic acid at 25oC. The electrolyte was used in the corrosion tests with or without the addition of rock rose floral (Helianthemum nummularium) at a concentration of 2.0 mL·L-1. The artificial saliva solution was used as the electrolyte, respecting the following composition: KCl 960 mg, NaCl 674 mg, MgCl2 41 mg, K2HPO4 274 mg, CaCl2 117 mg, D- sorbitol 24.0g, carboxymethyl cellulose 8.0g, completed with deionized water to a final volume of 1.0 L [26]. The pH was adjusted to 6.50 or 2.50 using sufficient lactic acid at 25oC. The electrolyte was used in the corrosion tests with or without the addition of rock rose floral (Helianthemum nummularium) at a concentration of 2.0 mL·L-1.

The electrochemical corrosion tests on the titanium samples consisted of 1) the open circuit potential measurements during 86,400s, one measurement every 60s 2) the electrochemical impedance spectroscopy (EIS) measurements performed at the corrosion potential of 8 mV sine wave perturbation in a frequency range from 20 kHz to 3 mHz with 10 points per frequency decade and 3) the potentiodynamic polarization with an applied potential scan rate of 0.30 mV·s-1. Initial potential started 0.25 V below the corrosion potential and the final potential was 2.0 V vs. Ag/AgCl reference. All measurements were repeated at least three times for each condition to obtain representa- tive results.

Statistical analysis

The presented statistical analysis consists of a factor analysis to verify if the pH and/or the presence of the floral influences the fol- lowing corrosion parameters: corrosion potential (Ecorr), corrosion current density (Jcorr), passivation current (Jpass) and polarization resistance (Rp). The analysis consists of an ANOVA 2 using two factors (pH and the presence of rock rose floral), with two levels each. The two levels for each factor were:

- Hydrogenionic potential: pH of 2.50 and 6.50;
- Rock rose floral: with and without floral.

For ANOVA 2 factor analysis, the following model describes each observation:

$$y_{ijk}=\mu+\tau_i+\beta_j+(\tau\beta)_{ij}+\varepsilon_{ijk}$$

where μ is the medium global effect, τ_i is the i-th effect of the factor A and β_j is the j-th effect of the factor B, the $(\tau\beta)_{ij}$ is the ij-th effect of interaction of the factors A and B. ε_{ijk} is a random error centered at zero.

The ANOVA 2 analysis verifies whether the factors: pH and floral presence or the interaction among them are significant for a given sta- tistical level. The significance evaluation can be based in a P-value obtained from F statistics, where a higher P-value than a chosen factor means that the given variable is significant. In this work, this factor was arbitrarily chosen as 0.05.

Results

In this paper, we essentially performed two types of tests, both important regarding factors in the onset of peri-implantitis. One test is the assessment of Staphylococcus aureus biofilm formation over pure titanium disks and the other is the electrochemical evaluation of titanium in artificial saliva with and without floral remedy.

Titanium surface treated with rock rose floral do not form Staphylococcus aureus biofilm

The in vitro biofilm formed on the titanium surface treated with rock rose floral had only 20% of the biofilm biomass regularly formed by the USA300 strain on pure titanium surfaces, indicating that the floral remedy interfered with the bacteria attachment, as shown in figure 1A.

Scanning electron microscopic observation of the titanium surface revealed the structure of the biofilm established by USA300 strain composed of bacterial microcolonies within a matrix. From biomass assay with floral treated titanium, visual evaluation of representative biofilm images appeared with a total biofilm volume significantly lower, looser and thinner on the surface. Taken together, these results strongly indicate that the rock rose floral remedy severely attenuated Staphylococcus aureus attachment on pure titanium surface. The surface attachment reduction is clearly noted in figure 1B.

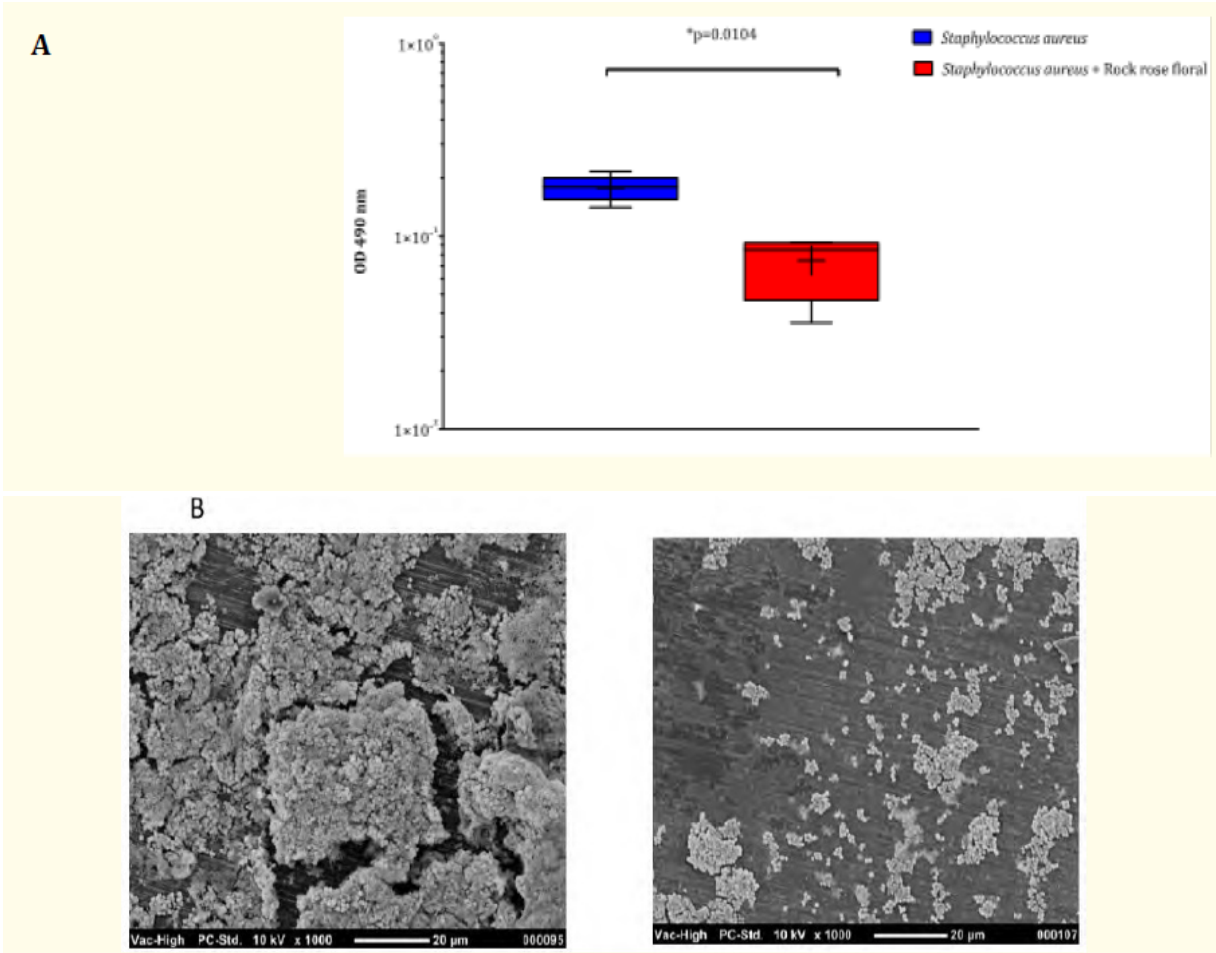

Figure 1: *In vitro biofilm assays for Staphylococcus aureus (USA300) on pure titanium surface. (A) Biomass quantification of biofilm formed on pure titanium. Pure titanium samples were affixed to bottom of microtiter plate, and biofilm allowed developing in TSB-G with 20% pooled human plasma. For A, representative images of biofilms after staining with crystal violet are shown at the bottom of x-axis, and biofilm mass was dissolved in acetic acid and quantified at optical density at 490 nm wavelength. P values were determined by unpaired Student's t test, with the Tukey multiple comparison post-hoc test. Staphylococcus aureus + Rock rose floral *, P = 0.0104 vs. Staphylococcus aureus. SEM images (B) of 24h biofilm on pure titanium samples and on floral treated titanium samples.*

Table Analyzed	Transform of USA300 versus Rock rose on titanium
Column B	Staphylococcus aureus + Rock rose floral
vs.	vs.
Column A	*Staphylococcus aureus* (USA300)
Unpaired t test	
P value	0.0001
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.7 df=11
How big is the difference?	
Mean ± SEM of column A	-0.7544 ± 0.02145, n = 9
Mean ± SEM of column B	-1.156 ± 0.09905, n = 4
Difference between means	-0.4012 ± 0.07037
95% confidence interval	-0.556 to -0.2463
R squared (eta squared)	0.7471
F test to compare variances	
F, DFn, Dfd	9.48, 3, 8
P value	0.0104
P value summary	*
Significantly different (P < 0.05)?	Yes

Supplementary data from figure 1A

Rock rose floral confers corrosion resistance to pure titanium

Figure 2 shows the representative results of the open potential versus time. As a general tendency, the potential increased as soon as the specimens were immersed in the artificial saliva solutions and then stabilized after 24h exposure in all cases. Higher open potential is a good indicator of a corrosion resistant response on the surface for a given medium. The steady-state value represents the corrosion potential.

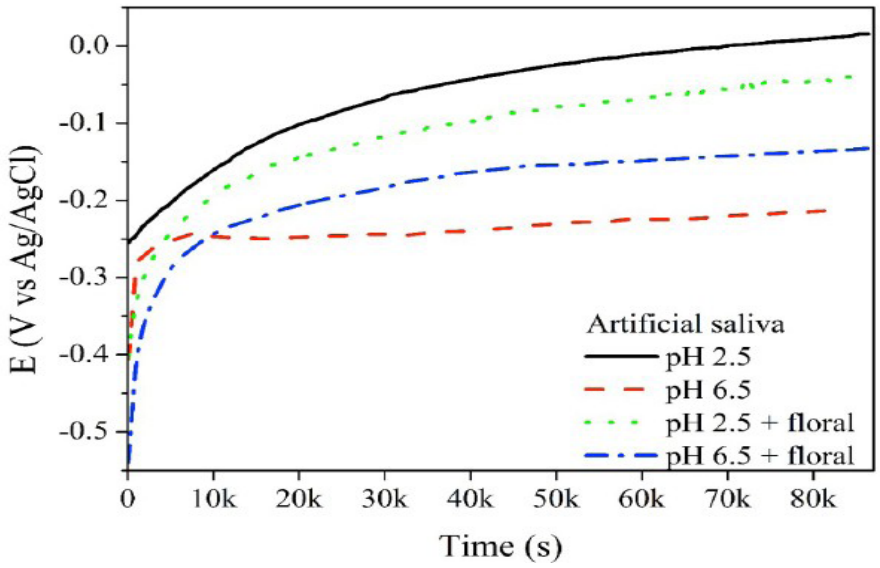

Figure 2: Evolution of open potential during 24h for the Ti-CP in the artificial saliva at different pH and in the presence and in the absence of the rock rose floral.

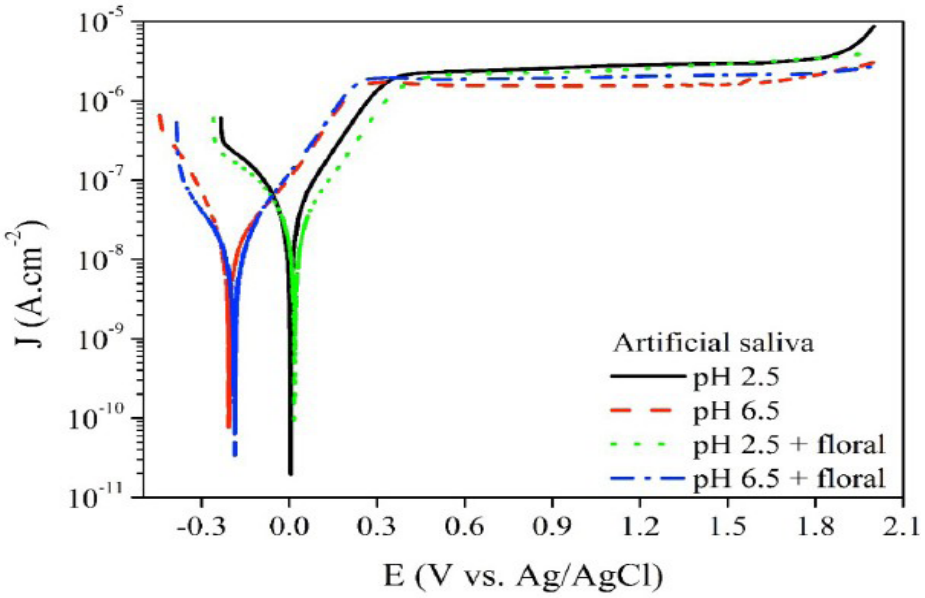

Figure 3: Potentiodynamic polarization curves obtained for Ti-CP in artificial saliva at 37°C, in two pH in the presence and in the absence of the rock rose floral.

Figure 3 presents the potentiodynamic polarization curves of Ti-CP in artificial saliva at different pH in the presence and in the absence of floral at 37°C.

The corrosion potential (Ecorr) and the corrosion current density (jcorr) obtained

by fitting the Tafel plots and passivation current (jpass) are shown in table 1.

Artificial saliva	pH	E_{corr} (V *vs.* Ag/AgCl)	J_{corr} ($\mu A \cdot cm^{-2}$)	J_{pass} ($\mu A \cdot cm^{-2}$)
without floral	6.50	-0.21 ± 0.02	0.026 ± 0.005	2.38 ± 0.48
	2.50	0.02 ± 0.03	0.180 ± 0.011	2.43 ± 0.34
with floral	6.50	-0.12 ± 0.02	0.016 ± 0.004	1.97 ± 0.07
	2.50	-0.02 ± 0.02	0.042 ± 0.008	2.48 ± 0.29

Table 1: Corrosion parameters of Ti-CP in the artificial saliva.

Corrosion parameters of Ti-CP in the artificial saliva at 37°C in two pH and in the presence and in the absence of rock rose floral. Results are presented as mean ± standard deviation.

The Jcorr increase in the acidified artificial saliva was reported in the literature [27,28], the obtained results were therefore expected. A statistical analysis with ANOVA 2 ($P < 0.05$) demonstrated that the rock rose floral presence improves corrosion resistance of Ti-CP in artificial saliva. The effect is accentuated in the acidified pH (Figure 4), which is an attempt to mimic the oral infection effect [29].

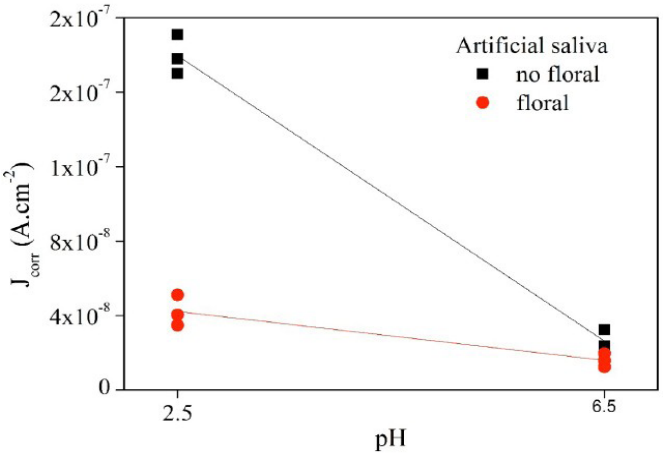

Figure 4: Effect of pH and rock rose floral on corrosion current density.

The reduction of pH increases the corrosion current density most likely because of the instability of the passive film. However, it is an important effect that the floral acts as a corrosion inhibitor at low pH. The corrosion is reduced to approximately a quarter of the artificial saliva without floral. Some interaction also can be observed at pH and rock rose floral because the lines are concurrent. In the normal condition of saliva, i.e. pH around neutrality, the rock rose floral has practically no effect because the film is very protective. Nonetheless, in low pH the rock rose floral, such as in the case of infection, the floral presents a positive response in regards to the corrosion of Ti-CP. The passivation current density does not show significant variations under different test conditions.

The EIS spectra in the Nyquist plot obtained at the corrosion potential for the different pH with and without the rock rose floral are presented in figure 5. The smaller

diameter of the semicircle observed with the artificial saliva at pH 2.50 was expected due to the aggres- siveness of the electrolyte, as was the larger diameter for the artificial saliva at pH 6.50.

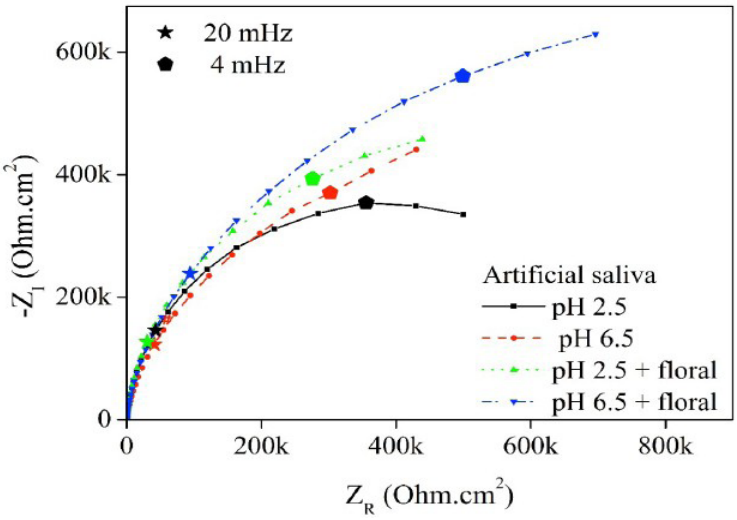

Figure 5: Nyquist plot for Ti-CP in the artificial saliva at 37°C in different pH and in the presence and absence of the rock rose floral.

An equivalent circuit model (Figure 6), which can be used to model passive layers [30], was applied in adjusting the experimental data. The model Rs represents the ohmic resistance of the solution and Rp stands for the polarization resistance whose value is a measurement of electron transfer across the passive surface. Although the accurate relationship between Rp and the corrosion intensity is complex in passive systems, an increase of Rp values to more corrosion resistant material is observed.

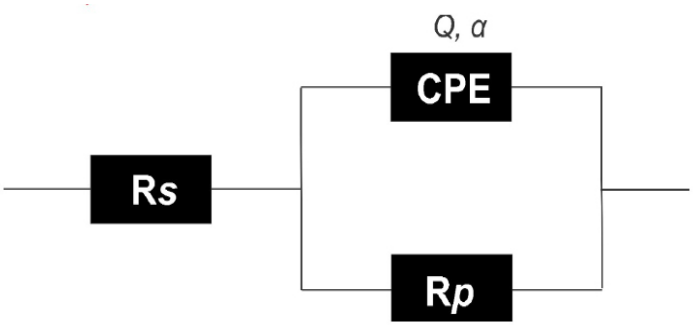

Figure 6: Equivalent circuit employed to fit the EIS data.

For passive alloys, the phase presents a large frequency span with high angle. To model this behavior, a simple combination of resis- tance and ideal capacitance is not adequate. To fit this type of EIS diagram, a Constant Phase Element (CPE) is ordinarily used to model the electrochemical impedance systems. This CPE impedance is defined as Z_CPE=1/[Q(iw)]^α , with −1 ≤ α ≤ 1. The constant iis the complex number (i^2=-1), and w is the angular frequency. The parameters α and Q are associated with time constant distribution of electrochemical processes. The constant phase element is introduced in the circuit instead of a pure double layer capacitor to improve the fitting

accuracy by

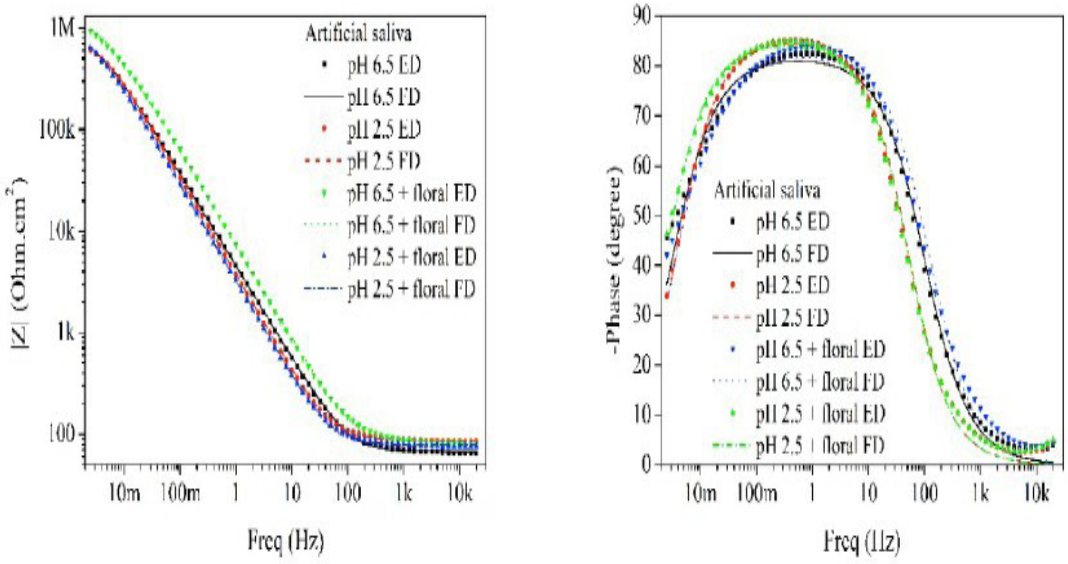

Figure 7: *EIS spectra (bode representation) for Ti-CP in artificial saliva at 37°C - experimental data (ED) and model fitted data (FD).*

the incorporation of surface heterogeneity to the model [31,32]. The α value is associated with the non-uniform distribution of current and potential related to surface defects. The fitted results are depicted in the curves of figure 7 as well as in the parameters of table 2.

	pH	CPE		R_p (MOhm·-cm^2)
		Q (μMho·cm-$^{-2}$.s$^{\alpha-1}$)		
without floral	6.50	32.4 ± 1.2	0.906 ± 0.008	2.07 ± 0.57
	2.50	43.9 ± 2.3	0.936 ± 0.029	0.89 ± 0.16
with floral	6.50	45.0 ± 5.7	0.936 ± 0.004	2.87 ± 0.39
	2.50	44.6 ± 1.4	0.952 ± 0.010	1.34 ± 0.35

Table 2: Electrochemical parameters from EIS plots for Ti-CP in the artificial saliva Electrochemical parameters obtained from EIS plots for Ti-CP in the artificial saliva at 37°C in dif- ferent pH and without or with rock rose floral. Results are presented as mean ± standard deviation).

DISCUSSION

Peri-implantitis, as osteomyelitis and others orthopedic implant infections, are considered a Staphylococcus spp. biofilm-associated infection. Although the complex mechanism required of the bacteria to form a functional biofilm is still under investigation, it is well known that the process is derived from the initial adhesion between bacteria and host tissue or even a biomaterial surface. Due to the capacity of Staphylococcus aureus to efficiently attach onto biomaterial surfaces, it can be detected on dental implant surfaces within an hour following surgical insertion [33].

In the present study, we observed that USA300, a community-associated

methicillin-resistant Staphylococcus aureus (CA-MRSA), formed a strong biofilm on pure titanium disks pre-coated with human plasma and that this biofilm formation was inhibited when rock rose floral remedy was added to the culture medium. Regarding Staphylococcus aureus, the adhesion step is an active process mediated by the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Examples include the clumping factor A and B (clfA, clfB), fibronectin binding protein A and B and serine-aspartate repeat protein-encoding C, D and E (sdrCDE) proteins which are covalently catalyzed anchoring to the cell wall by a sortase (A) enzyme [25,34].

The mechanism by which rock rose floral remedy reduced USA300 biofilm formation on titanium could be either related to inhibition of sortase A proteinaceous biofilm or just because of its antibacterial effect. To this end, our preliminary results have shown no differences between the growing curve of USA300 in presence or in absence of rock rose floral remedy (data not shown).

Titanium-based implants are widely used in modern clinical practice but their “optimal” properties in terms of porosity and topology as well as their roughness and hydrophilic parameters, are a subject of intense debate. Recent in vitro results have shown a possibility to optimize the surface of an implant with maximal repelling of bacteria (Staphylococcus aureus, Staphylococcus epidermidis) and improve- ment in human osteogenic and endothelial cell adhesion, proliferation and differentiation [35-37].

Healthy peri-implant tissue plays an important role as a biological barrier to the agents that cause peri-implant disease [38]. Low pH produced by inflammation response and the bacteria biofilm formation expose the titanium to corrosion-avoiding osteointegration. We confirmed that the acid pH reduces the Rp value, indicating that in these conditions the corrosion is more intense. The presence of the rock rose floral remedy enhances this parameter showing that the corrosion resistance of the pure titanium increased in this medium. The ANOVA 2 shows that the two-factor analysis presents a synergic effect on corrosion current density, passivation current density and polarization resistance (Table 3).

P-values	J_{corr}	J_{pass}	R_p
pH	+++	-	++
Floral	+++	-	+++
Interaction	+++	-	-

Table 3: Statistical analysis results for Jcorr, Jpass and Rp.

+++ strong (P-value < 0.01); ++ medium (P-value < 0.05); - weak (P-value > 0.1).

These facts reinforce the hypothesis that the floral act strongly on corrosion current density and polarization resistance. Additionally, the pH and the floral exhibit

interaction. This interaction can be understood as a synergy, in the sense that the increase of pH, from 2.50 to 6.50, and adding the floral, reduces the corrosion current density in artificial saliva. In other words, under physiological conditions, the corrosion intensity of pure titanium is inferior than under pathogenic status. The passivation current density, on the other hand, does not change regardless the pH and floral.

CONCLUSION

Within the limitations of the present study, it can be shown that rock rose remedy prevented bacteria adhesion over the pure titanium. Moreover, in artificial saliva similar to a healthy oral environment (pH 6.50), it did not seem to interfere with the titanium corrosion re- sponse. The electrochemical parameters related to corrosion behavior worsen at pH 2.50 in comparison to pH 6.50, but interestingly the presence of rock floral (Helianthemum nummularium) reduces the loss of corrosion resistance, acting as a natural corrosion inhibitor in low pH. In summary, rock rose floral reduces the bacterial attachment in a normal saliva environment and increases the corrosion resistance of commercially pure titanium in acidified buccal environments.

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