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Evaluation of chlorhexidine /polydopamine antimicrobial coatings on the Ti-7.5 Mo alloy surface - in vitro studies

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Abstract *— Titanium and its alloys are widely used in biomedical applications because of their excellent properties such as high corrosion resistance, biocompatibility and mechanical properties. One of the applications is in dental implants and usually presents failures due to infectious processes caused by the formation of a biofilm layer on the implant surface. The biofilm structure makes it difficult to treat these infections, leading to the development of new surface modification techniques, in order to prevent the adhesion of microorganisms to the implant surface. The objective of this study was to evaluate the efficiency of a Ti7.5Mo alloy surface coating with a natural polydopamine (PDA) polymer associated with a bactericidal agent, chlorhexidine (CHX). The surfaces were characterized using contact angle (AC) and surface energy. Cytotoxicity using adipose derived stem cells and antibacterial behavior using Staphylococcus aureus and Candida albicans were evaluated. Both groups coated with this antibacterial system acted efficiently in combating the formation of C. albicans and S. aureus biofilms without causing cytotoxic effect on stem cells derived from adipose tissue. Therefore, the coating of the Ti-7.5Mo alloy with polydopamine and chlorhexidine is a promising application for biomedical devices aimed at reducing the formation of biofilms and, consequently, the reduction of postoperative infections.*

Keywords *— **Titanium alloy; Nanotube TiO_2; Chlorhexidine; Polydopamine.***

I. INTRODUCTION

Among the available materials for dental implants application stand out titanium (Ti) and its alloys, due to its excellent biocompatibility and mechanical properties such as high strength, low density and high corrosion resistance [1]. However, one of the major causes of implants failure is related to the biofilm formation and infectious processes. The biofilm structure complicates the treatment of these infections [2]. At the time of implant introduction, bacteria such as *Staphylococcus aureus* and *Escherichia coli* can adhere and proliferate on its surface and forming a biofilm [3]. Immediately after the implant introduction, a layer formed by proteins such as fibrinogen, fibronectin and collagen, covers the implant surface and promotes the floating bacteria adherence, which form a biofilm later. The biofilm infection is difficult to treat because biofilm blocks the macrophages penetration and the systemic antibiotics, promoting greater bacterial survival [4].

Currently there is a growing interest in research aimed at functionalizing titanium surfaces with a focus on accelerating bone healing and preventing bacterial adhesion. The intention is to reduce the bacterial colonization likelihood of the implant surface during surgery [2, 5]. Several studies related to the development of antibacterial coatings on metal surfaces have already been performed and have effectively eliminated the infection and biofilm formation [6, 7]. Among the antibacterial agents studied are chitosan, silver, and chlorhexidine [8, 9, 10, 11, 12].

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Chlorhexidine is an antiseptic compound with high antibacterial action, with an effective action against a wide variety of gram-positive and gram-negative microorganisms, besides inhibiting the action of some fungi and enzymes [13]. In dentistry, chlorhexidine is used in various oral hygiene products such as mouthwashes, toothpastes, topical antimicrobial agents and is also used for general skin cleansing and as a pre-operative preparation. Its antibacterial action can be attributed to a rupture of the bacterial cell membrane, which interferes with the adhesion of this bacteria. Due to its cationic nature, it is highly significant in oral tissues, leading to an electrostatic attraction with the various oral surfaces that carry negative charge [14]. Chlorhexidine and its water soluble derivative, chlorhexidine digluconate, have been widely used to control biofilms in teeth, being so far the most effective treatment due to its several advantages: high antimicrobial capacity and ability to inhibit glycosidic and proteolytic activities and reduce the action of matrix metalloprotease in most oral bacteria. Chlorhexidine is also retained by dentin hard tissues and its is an effective irrigator to prevent root canal reinfection due to coronal leakage [2].

In addition to these antibacterial coatings, titanium coatings with a polymeric material for the drugs controlled release are indicated as a possible solution to prevent biofilm growth. Between these polymers include the polydopamine with excellent properties of biocompatibility and biodegradation, besides high elasticity modulus and hardness [15]. This polydopamine is spontaneously formed by the oxidation of dopamine in alkaline solutions and has a chemical binding mechanism based on its amine and catechol functional groups that are responsible for the continued release of any drug incorporated into it [16]. These polydopamine coatings may be used for the the drugs controlled release as hollow permeable polydopamine capsules for small molecules or incorporation of the drug directly into the film [15].

The objective of this study was to evaluate the efficiency of a Ti7.5Mo alloy surface coating with a natural polydopamine (PDA) polymer associated with a bactericidal agent, chlorhexidine (CHX). The surfaces were characterized using Fourier-transform infrared spectroscopy (FTIR), contact angle (AC) and surface energy. Cytotoxicity using adipose derived stem cells and antibacterial behavior using *Staphylococcus aureus* and *Candida albicans* were evaluated.

II. MATERIALS AND METHODS

2.1 PROCESSING OF THE SAMPLES

To obtain the Ti-7,5Mo alloy, commercially pure metals were used in sheets: titanium CP (Sandinox) and molybdenum 99.99% (Sigma Aldrich). The Ti7.5Mo alloy was melted in an arc furnace with an inert atmosphere, copper crucible cooled with water. The ingots were homogenized under vacuum at 1100ºC for 24 h to eliminate chemical segregation and were cold-worked by swaging (FENN), producing an 8 mm rod. Due to the hardening caused by forging, the bars were subjected to the solubilization heat treatment at 950 ° C for 2 hours, followed by rapid cooling in water. Discs with 3 mm thickness were cut on the Isomet 4000. The samples were sanded with silicon carbide sandpaper with granulation up to 1200, polished with colloidal silica, washed with deionized water and immersed in an ultrasonic bath with anhydrous alcohol for 5 minutes.

The samples were divided into three groups: sanded (S), coated with polydopamine associated chlorhexidine (ACH) and coated with polydopamine and immersion in chlorhexidine (ICH). A solution of 2.0 g / l polydopamine in 10 mM Tris-HCl, pH 8.6 was prepared. For the group (ACH), 10 ml of chlorhexidine (2%) were added to the polydopamine solution in a 1: 1 ratio, stored for 24 hours in a dark environment to avoid degradation by light and dried for 24 hours at room temperature. For the group (ICH), the samples were immersed in this polydopamine solution for 24 hours, dried for 24 hours in a desiccator, immersed in 10 ml of chlorhexidine (2%) for 30 minutes and dried at room temperature.

2.3. SURFACE CHARACTERIZATION

The wettability characteristics of the surface were evaluated by contact angle using the sessile drop technique, on a Ramé-Hart 300-F1 Goniometer. The experiments were carried out in ambient conditions, considering the average value of three measurements, at different places in the samples. The surface energy was calculated using two different liquids, deionized water (polar liquid) and ethylene glycol (apolar liquid) using DROP image software.

2.4 CYTOTOXICITY ANALYSIS

2.4.1 ADIPOSE DERIVED STEM CELL CULTURE

Adipose Derived Stem Cells were donated by Dr. Kimberly Cox-York from the department of Food Science and human Nutrition at Colorado State University. The cells were cultured at 37 ºC and 5% CO_2 in growth media consisting of MEM-Alpha Modification Media, GE Life Science-Hyclone with 10 % of Fetal Bovine Serum (FBS)

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and 1% of penicillin/streptomycin (Sigma). The growth media was changed every other day. To the tests on surfaces the cells were detached using 0,25% Trypsin-EDTA, centrifuged at 1000 rpm for 10 mins. Following centrifugation the cells were counted at concentration of $1x10^4$, using Neubauer chamber and Trypan Blue. Cells were seeded on all surfaces in 24 well plates.

2.4.2. TOXICITY

Cytotoxicity was investigated after 1 day of initial culture using Lactate Dehydrogenase-LDH Cytotoxicity Assay Kit (QuantiChrom). Cytotoxic compounds often compromise cell membrane integrity by inducing apoptosis or necrosis. LDH is a stable cytosolic enzyme that with membrane damage is released into the cellular environment. Therefore, LDH is often measured to evaluate the presence of tissue or cell damage. The cells were incubated at 37 °C for 4 h in α-MEM and 160 µl of reagent per well. After 4 h, 100 µl of supernatant was transferred into a 96-well plate. The optical density of solution was measured at 500 nm using a spectrophotometer (FLU Ostar Omega; BMG Labtech, Durham, NC).

2.4.3 CELL VIABILITY

The cell viability was measured after 1 and 7 days of culture using Alamar Blue Assay Reagent (Promega). Adhered cells were incubated at 37 ºC for 4 hrs in fresh α-MEM and 10% of Alamar Blue Reagent. The Alamar Blue Reagent is an oxidized form redox indicator that is blue in color. When incubated with viable cells, the reagent changes color from blue to red. After 4 hrs the optical density (OD) of solution was measured at 570 nm and 600 nm using a spectrophotometer (FLUO-star Omega; BMG Labtech). The percentage reduction of Alamar Blue was calculated as described by the company instructions.

2.4.5. CELL ADHESION AND PROLIFERATION

After 1 and 7 days of initial culture the cell adhesion and proliferation were investigated by fluorescence staining with Rhodamine Phalloidin (actin-cytoskeleton) at 70 nM, and 4' 6-diamidino-2-phenylindole DAPI (nucleus) at 300 nM. The surfaces were removed from the growing media, washed with PBS and fixed with 3,7% of formaldehyde for 15 mins at room temperature. To permeabilize the cells, the surfaces were incubated with 1% of Triton-X100 for 3 mins, and then washed with PBS. The surfaces were incubated in rhodamine-phalloidin stain at a concentration of 70 nM for 30 mins at room temperature. After 25 mins of rhodamine-phalloidin staining, DAPI was add at concentration of 300 nM for 5 mins. All the solution was aspirated and the surfaces were then washed with PBS and imaged using a Zeiss Imager-A2 fluorescence microscope. The number of adhered cells on the surfaces was determined from 10X DAPI stained images by counting the nuclei. These analysis were performed using "Analyze Particles" feature embedded in the ImageJ software. The cell coverage was determined from 10X Rhodamine Phalloidin stained images. These analysis were performed using "Masks" feature embedded in the ImageJ software.

2.5 ANTIBACTERIAL ANALYSIS

The antibacterial action of cloroxidine-impregnated samples were evaluated against *Candida albicans* (ATCC 18804) and *Staphylococcus aureus* (ATCC 35688). For each strain a standard suspension with optical density equivalent to 106 cells / ml was prepared. *C. albicans* strains were seeded on Sabouraud agar (Difco, Detroit, USA) and *S. aureus* on brain / heart infusion (BHI) agar (Difco, Detroit, USA) and incubated at 37øC for 24 h .

After the incubation time, the cells were suspended in sterile saline (sodium chloride 0.9% (NaCl)) and counted in a spectrophotometer (B582, Micronal, São Paulo, Brazil). The optical density and wavelength parameters used were 0.284 and 530 nm for *C. albicans*; and 0.620 and 398 nm for *S. aureus*.

Previously to biofilm growth, the samples were sterilized and incubated at 37 °C for 48 hours at partial pressure 5% CO_2, with 2 ml Brain Heart Infusion broth (BHI, Difco, Detroit, USA) supplemented with 5% sucrose and inoculated with 0.1 ml of the microbial suspension to be analyzed.

After the incubation period, samples containing the biofilms were washed twice with 2 ml of 0.9% NaCl and sonicated (Sonoplus HD 2200, 50 W. Bandelin Eletronic, Berlin, Germany) for 30 seconds to disperse the biofilms. Biofilm suspensions were serially diluted in 0.9% NaCl to give dilutions of 10^{-1} to 10^{-5} times the original concentration.

Aliquots of 100ul of each dilution were seeded in duplicate in Sabouraud Agar (Difco, Detroit, USA) with 50 mg / L chloramphenicol for *C. albicans*; and Mitis Salivarius agar (Difco, Detroit, USA) supplemented with 0.2 IU / ml of bacitracin (União Química, São Paulo, Brazil) and 15% sucrose for *S. aureus*. The plates were placed in a bacteriological incubator for 48 hours at 37 °C. After incubation for 48 h, the number of colony-forming units per milliliter (CFU / ml) was determined. The results were log-transformed (log_{10}) and analyzed by analysis of variance (ANOVA) and Tukey's test.

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III. RESULTS AND DISCUSSION

3.1. SURFACE CHARACTERIZATION

To study the effect of polydopamine and chlorhexidine films on the wettability of the Ti-7.5Mo alloy surface, contact angle and surface energy measurements were performed. Hydrophobic surfaces have a contact angle greater than 90 °, while on hydrophilic surfaces this angle is less than 90 °.

Table 1 shows the contact angle and surface energy values. It is observed that the sanded sample had a higher contact angle (102.92 °) and a hydrophobic behavior. After the polydopyma and chlorhexidine coating, the contact angle decreased and the wettability changed, making the ACH surface hydrophilic (61.27 °) and the super hydrophilic ICH surface (43.92 °). This super hydrophilicity of the ICH surface occurred due to the exposure of the Ti-7.5Mo substrate for 24 hours only the polydopamine, since the chlorhexidine was added after these 24 hours of polymerization. The surface energy for the ACH samples (47.64 ± 0.24 mJ m-2) and for ICH (57.72 ± 0.34 mJ m^{-2}) show a polar behavior, which is very promising for biomedical applications. It has been reported that polydopamine is capable of transforming hydrophobic surfaces into hydrophilic ones [17, 18]. According to Ball et al., (2011) [19] the complex structure of a polydopamine film on a metallic substrate or hydrophobic increases water penetration and renders the surface more hydrophilic. In another study, on hydrophobic substrate the surface energy of polydopamine was also evaluated and demonstrated that polydopamine coatings are essentially of polar nature and hydrophilic [20].

Table 1 – Contact angle and surface energy of samples

Sample	Contact angle (°C)		Surface energy
	Deionized water	Ethyleneglycol	
Ti7.5Mo - S	102.92 ± 0.51	84.37 ± 0.26	18.31 ± 0.11
Ti7.5Mo - ACH	61.27 ± 1.48	9.18 ± 0.09	47.64 ± 0.24
Ti7.5Mo - ICH	43.92 ± 0.62	17.61 ± 0.03	57.72 ± 0.34

3.2. CYTOTOXICITY ANALYSIS

The ability of the substrates to stimulate adhesion and proliferation was assessed by fluorescence analysis and labeling of the cell nucleus with DAPI. Figure 1a shows the nuclei DAPI-labeled and blue-stained, and the actin filaments present in the cytoskeleton labeled with Rhodamine-phalloidin and stained red. The image shows cells adhered to the substrate, with well preserved and elongated cytoskeleton on all treatments. Figure 1b shows the quantitative analysis of the number of cells by counting the nuclei labeled with DAPI. It was possible to verify that there was a significant proliferation of day 1 to day 7 of the ADSCs incubated with the substrate Ti 7.5 Mo-S. Treatments Ti 7.5 Mo-ACH and Ti 7.5 Mo-ICH maintained the same number of cells when we compared day 1 and day 7. Study of Swiatkowska et al 2016, demonstrates that human cells may have suppressed growth when in contact with clorexidine, data that corroborates with our study [21].

The cytotoxicity test was performed by measuring the Lactate Dehydrogenase (LDH) present in the medium. LDH is an enzyme that is released in the medium when the membrane is damaged. Figure 2a shows that the different treatments did not provide an increase in LDH release in the medium, indicating that the substrates were nontoxic to the ADSCs.

Viability was analyzed using the Alamar Blue test. This test evaluates the ability of viable cells to metabolize the reagent [22,23]. Figure 2b shows that there was no significant difference in the metabolization of the reagent by cells on the different substrates on day 1, indicating a homogeneous viability between treatments. On day 7 there was a significant decrease in cellular activity for treatments Ti 7.5 Mo-ACH and Ti 7.5 Mo-ICH compared to the Ti 7.5 Mo-S. However, when we compared day 1 and day 7, the cells maintained the same behavior for treatments Ti 7.5 Mo-ACH and Ti 7.5 Mo-ICH. Together the tests of LDH and Alamar Blue demonstrate that besides being nontoxic, the substrates allow the maintenance of the ADSCs in all treatments. These data corroborate with the result of cell count.

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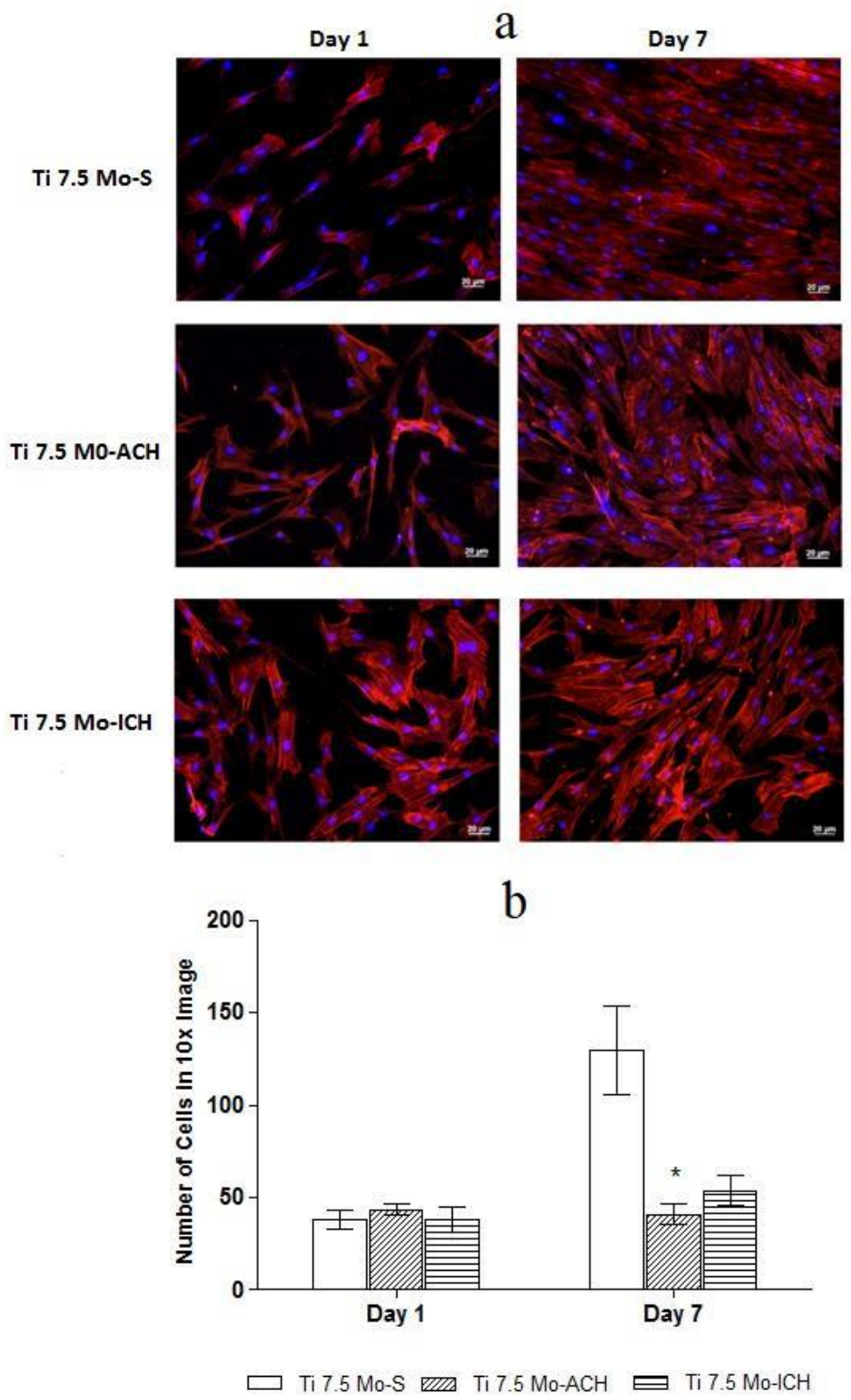

Fig.1 - Representative fluorescence images (10x) of ADSCs stained with DAPI and rhodamine/phalloidin on on Ti 7.5 Mo-S, Ti 7.5 Mo-ACH and Ti 7.5 Mo-ICH after 1 and 7 days of culture (a); ADSC counts on Ti 7.5 Mo-S, Ti 7.5 M0-ACH and Ti 7.5 Mo-ICH after 1 and 7 days of culture. The data presented is mean ± SD. (*) $p < 0.05$ using one-way ANOVA test and Tukey HDS to multiple comparisons, SPSS 13.0 software (b).

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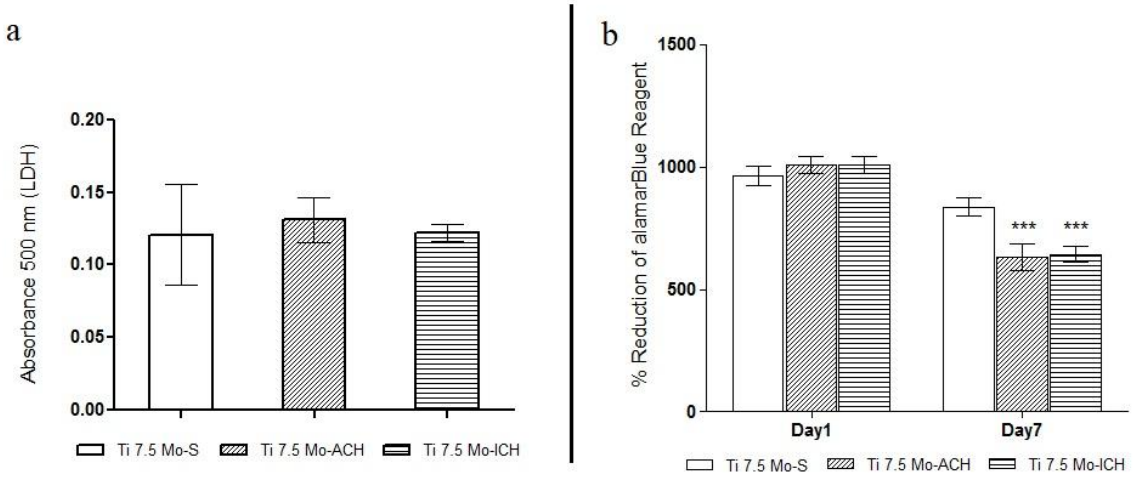

Fig. 2 - Cell toxicity measured by LDH assay on Ti 7.5 Mo-S, Ti 7.5 Mo-ACH and Ti 7.5 Mo-ICH 1 day of culture. The data presented is mean ± SD using one-way ANOVA test and Tukey HDS to multiple comparisons, SPSS 13.0 software (a); Cell Viability measured by Alamar Blue Cell Viability Assay on Ti 7.5 Mo-S, Ti 7.5 M0-ACH and Ti 7.5 Mo-ICH after 1 and 7 days of culture. The data is presented as mean ± SD. (***) p < 0.001 using one-way ANOVA test and Tukey HDS to multiple comparisons, SPSS 13.0 software (b).

3.3. ANTIBACTERIAL ANALYSIS

Bacterial adhesion was evaluated by the multi-species biofilm growth (*Candida albicans* and *Streptococcus aureus*) for 48 hours. Figure 3 shows the scanning electron microscopy image with the formation of the multi-species biofilm on the surface of the samples studied.

Figure 3 shows the chlorhexidine action effect incorporated in the sample surface in the C.Albicans and S. aureus biofilms formation. On theTi7.5Mo - S sample surface it is possible to visualize the dense and continuous biofilm formation, with agglomerated colonies covering the entire surface (Figure 3a). For the samples coated with PDA and CHE, the biofilm formation is visibly smaller. The Ti7.5Mo - ICH samples (Figure 3c) showed a surface with a dispersed and less dense biofilm compared to the Ti7.5Mo - S samples, while the Ti7.5Mo - ACH samples (Figure 3b) presented a lower biofilm formation among the investigated conditions, with smaller and more dispersed colonies.

By the analysis of scanning electron microscopy images, there is a reduction in biofilm formation for both Ti-7.5Mo-ACH and Ti-7.5Mo-ICH groups (Figure 3 (b) and 3 (c)) and these results corroborate Cortizo et al (2012) [2] which demonstrated that the addition of small chloroxidine doses in polymer coatings causes a reduction in the biofilm layer. In addition, Toté et al 2010 [24] reported that the viability of S. aureus biofilms is reduced by 84% in the presence of chlorhexidine and the cells response to the antimicrobial treatment is related to their location within the biofilm structure. Less dense biofilms present less heterogeneous responses, and the eradication of this biofilm is easier.

As an antiseptic compound, the chlorhexidine is capable to inhibit the glycosidic and proteolytic activity, action of several matrix metalloproteinases and the activity of bacterial collagenase. In addition, it has a high antibacterial action, with an effective action against a wide variety of gram-positive and gram-negative microorganisms, attacking the cytoplasmic contents and allowing the intracellular compounds to escape, which finally leads to bacterial death, besides inhibiting the action of some fungi and enzymes [25,26].

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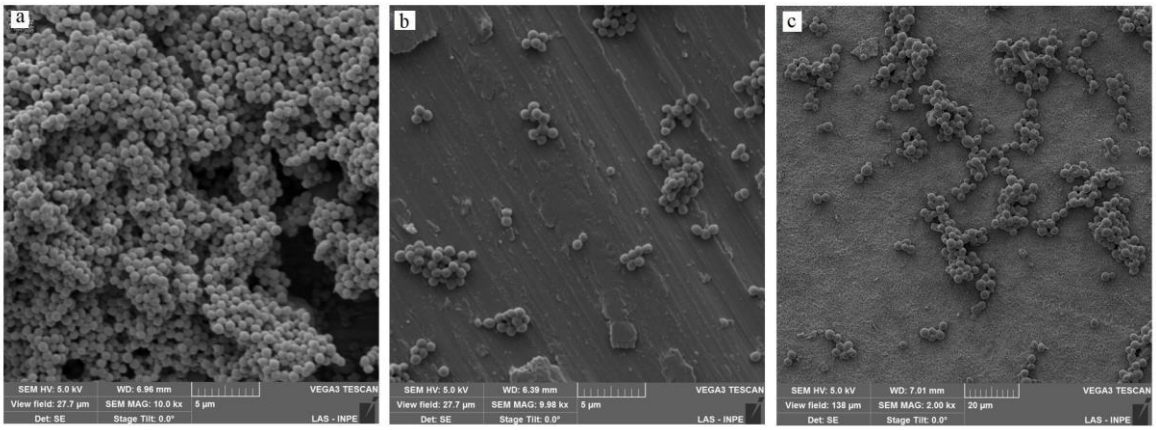

Fig.3 - Scanning electron microscopy with the biofilm formation of S. aureus and C. albicans on the samples surface studied. Ti-7.5Mo-S (a); Ti-7.5Mo-ACH (b); Ti-7.5Mo-ICH (c).

The antibacterial action of the samples impregnated with chlorhexidine was evaluated by determining the number of colony forming units per milliliter (CFU / ml) of C. albicans and S aureus. The results were log-transformed (log10) and analyzed by analysis of variance (ANOVA) and Tukey's test.

The mean values and standard deviation of the CFU / ml (log10) obtained in the experimental conditions tested for each biofilms group are shown in Table 2.

The mean values for *C. albicans* and *S. aureus* indicate that Ti-7.5Mo-ACH group presented a better result in relation to the others. However, to assert this result with greater security, a hypothesis test was performed considering that the average of CFU / ml for three groups are the same (H0: μI = μII = μIII). The null hypothesis (H0) was tested by analysis of variance (ANOVA) and the Tukey test.

Table 2 – Means values (CFU log10) and p values obtained for the biofilm

Group	**Microbial biofilms CFU log^{10}**			
	C. albicans	p value *	*S.aureus*	p value *
Ti-7.5Mo-S	6.933	0.703	7.461	0.919
Ti-7.5Mo-ACH	5.466	0.746	3.305	0.813
Ti-7.5Mo-ICH	6.726	0.355	6.866	1.605

Tables 3 and 4 indicate the differences between the means of the groups and the value P. It was considered that a P value <0.05 indicated a significant statistically difference.

The lower P value, the greater security in denying H0. We can say with a high level of reliability that for both microorganisms the Ti-7.5Mo-ACH group differs from Ti-7.5Mo-ICH and Ti-7.5Mo -S, with a lower average CFU. Therefore, the surface coating with polydopamine and associated chlorhexidine (Ti-7.5Mo-ACH) resulted in a significant decrease in biofilm growth for *C. albicans* and *S. aureus* both groups. However, the treatment with polydopamine immersed in chlorhexidine (Ti-7.5Mo-ICH) presented an lower average than the control group (Ti-7.5Mo-S), but this difference is not statistically significant.

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Table 3 – Tukey Test of S. aureus biofilm

Ti-7.5Mo-S		
	Averages difference	*P* value
Ti-7.5Mo-ACH	-4.156 ± 0.5831	0.0000
Ti-7.5Mo-ICH	-0.595 ± 0.5831	0.5725
Ti-7.5Mo-ACH		
	Averages difference	P value
Ti-7.5Mo-ICH	-3.561 ± 0,5831	0.0000

Table 4 – Tukey Test of C. albicans biofilm

Ti-7.5Mo-S		
	Averages difference	*P* value
Ti-7.5Mo-ACH	-1.371 ± 0,3128	0.0007
Ti-7.5Mo-ICH	-0.148 ± 0,3331	0.8972
Ti-7.5Mo-ACH		
	Averages difference	P value
Ti-7.5Mo-ICH	-1.223 ± 0,3244	0.0031

IV. CONCLUSION

In this work, it was possible to perform the incorporation of chlorhexidine on the Ti-7.5Mo alloy surface by polydopamia polymerization. This coating allowed an increase in the surface wettability, making it hydrophilic. Both groups coated with this antibacterial system acted efficiently in combating the formation of C. albicans and S. aureus biofilms without causing cytotoxic effect on stem cells derived from adipose tissue. Therefore, the coating of the Ti-7.5Mo alloy with polydopyma and chlorhexidine is a promising application for biomedical devices aimed at reducing the formation of biofilms and, consequently, the reduction of postoperative infections.

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