UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

OSCAR OSWALDO MARCILLO TOALA

**Anti-Adherent and Antifungal Activity of Synthetic and Organic
Adhesive Polymers on *Candida albicans* biofilms**

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Adhesive Polymers on *Candida albicans* biofilms**

**Efeito antifúngico e antiaderente de adesivos sintéticos e orgânicos
no desenvolvimento de *Candida albicans***

Dissertação apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Reabilitação Oral (Linha de pesquisa: Prótese).

Orientador: Prof. Dr. Vinicius Carvalho Porto

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Dedicatória

Aos meus pais: Mario Oswaldo Marcillo Baque e Guadalupe Annabell Toala Salazar; e meu irmão: Christian Andrés Marcillo Toala

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ABSTRACT

ANTI-ADHERENT AND ANTIFUNGAL ACTIVITY OF ORGANIC AND SYNTHETIC ADHESIVE POLYMER ON CANDIDA ALBICANS BIOFILMS

The objective of this study was to analyze the effect of synthetic and organic adhesive on *Candida albicans* biofilms. Prevention and management of *Candida*-associated denture stomatitis have been widely studied but still looking for an irrefutable mechanism. In the last years, the innovative application of Cyanoacrylates and Fibrin Sealant derived from Snake-venom with antimicrobial purposes has caught attention in medical fields. These coating materials could act directly on the applied surface causing cytopathic or cytotoxic effects on cells and microorganisms. Fibrin sealant and cyanoacrylates with different hydroxyl chains were coated on rectangular specimens (2-3 μ m Ra) and inoculated with *Candida albicans* SC5314 (1.10⁷ cells/mL) for 24 h. They were submitted to colony-forming units (CFU/mL) quantification assay, Colorimetric assay XTT and one specimen of each group was analyzed using Confocal Laser Scanning Microscopy for a qualitative analysis. The data was analyzed using Kruskal-Wallis and Dunn's multiple comparisons tests (p<0.05). According to the CFU assay a growth reduction was observed in Octyl-Cyanoacrylate, Conventional Ethyl-Cyanoacrylate, Gel Ethyl-Cyanoacrylate and Butyl-Cyanoacrylate groups with 87, 84, 71 and 60 % respectively. The XTT assay demonstrated a decreased metabolic activity in Octyl-Cyanoacrylate (70%) and Conventional Ethyl-Cyanoacrylate (61%). The Confocal Images showed a reduced fungal colonization in all Cyanoacrylate groups with no sign of non-viable adhered cells on Octyl-Cyanoacrylate. Based on these findings, an anti-adherent effect against *Candida albicans* can be achieved by coating Octyl-Cyanoacrylate and Conventional Ethyl-Cyanoacrylate on heat-polymerized acrylic resin. Furthermore, more tests must be held on in order to understand the anti-fungal effect.

Key words: *Candida albicans*. Adhesives. Tissue adhesives. Denture Bases.

RESUMO

Efeito antifúngico e antiaderente de adesivos sintéticos e orgânicos no desenvolvimento de *Candida albicans*

O objetivo do trabalho foi avaliar o efeito de adesivos sintéticos e orgânicos no desenvolvimento e adesão da *Candida albicans*. A prevenção e tratamento da estomatite associada à *Candida* tem sido estudada amplamente nos últimos anos, mas ainda procura-se um mecanismo irrefutável. Nos últimos anos, a aplicação de Cianoacrilatos e Selante de Fibrina derivado de veneno de cobra com finalidade antimicrobiana tem chamado à atenção dos pesquisadores e clínicos. Estes materiais quando aplicados na superfície podem exercer um efeito citopático ou citotóxico nas células humanas e microrganismos. Cianoacrilatos com diferentes cadeias e o selante de fibrina foram aplicados sobre espécimes quadrangulares (2-3µm Ra) e inoculados com *Candida albicans* SC5314 (1.10^7 cels/mL). Após 24 horas, os espécimes foram submetidos a ensaio de contagem de colônias fungicas (CFU/mL), ensaio de colorimétrico (XTT) e avaliação de imagens pela microscopia confocal. Os testes estatísticos empregados foram Kruskal Wallis e Dunn comparações multiples com $p < 0,05$. Os resultados demonstraram uma redução da contagem de colônias nos grupos cianoacrilatos: Etil-cianoacrilato Convencional (84%), Etil-cianoacrilato gel (71%), Butil-cianoacrilato (60%) e Octil-cianoacrilato (87%). O teste XTT apresentou os maiores resultados nos grupos Octil-cianoacrilato (70%) e Etil-cianoacrilato Convencional (61%). As imagens obtidas na microscopia confocal confirmaram uma redução da adesão fungica de células viáveis e não viáveis nos grupos cianoacrilatos compatível com o CFU/mL; principalmente no grupo Octil-cianoacrilato onde só tinha células viáveis aderidas. Estes achados confirmaram a atividade antiaderente dos cianoacrilatos contra a colonização de *Candida albicans*. A incorporação de mais períodos de avaliação assim como outros testes tem que serem desenvolvidos para compreender como esse efeito antiaderente acontece.

Palavras chaves: *Candida albicans*. Adesivos. Adesivos Teciduais. Base de dentadura.

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1 Introduction

1 INTRODUCTION

Oral diseases have been studied for many decades describing the pathogenesis of each disease and how they could be treated and prevented. The most common human oral disease in edentulous is the Denture Stomatitis, which could be initiated by an allergic factor as well as microbial colonization. The microorganisms frequently isolated are *Candida* spp., which colonize oral and vaginal mucosae. Therefore, this fungal mucosal alteration is recognized as *Candida*-associated denture stomatitis (GENDREAU; LOEWY, 2011; SOLLECITO; STOOPLER, 2013; STOOPLER; SOLLECITO, 2014).

Candida albicans, present in more than 90% of healthy individuals but in some environmental conditions, it could initiate a pathogenic state. This state requires a multifactorial etiology that could vary from compromised autoimmune response, poor hygiene, prosthetic material properties, host susceptibility, oral microbiological interactions and continuous denture wearing. *Candida albicans* can adhere and develop to any acrylic surface and initiate a tissue invasion when its genetic virulence factors are expressed (FARAH; LYNCH; MCCULLOUGH, 2010; MUZYKA; EPIFANIO, 2013; SHAFREEN; MUTHAMIL; PANDIAN, 2014).

Candida's adherence is an interrelated mechanism of strong and weak binding forces, which could make the adhesion reversible or irreversible due to the specific force interaction. The key point relies on the distance the fungi can approximate to the matrix or substrate; studies indicate a mean separation of 10 nm could turn into an irreversible attachment carried out by fungal Adhesines (strong binding forces) produced to interact with matrix proteins. Other forces contribute to the adherence as the cell surface hydrophobicity force and cell zeta potential (MCCARRON et al., 2004; MCCARRON et al., 2007; PEREIRA-CENCI et al., 2007).

On the acrylic resin, *Candida albicans* biofilm formation starts with an initial adhesion to the acrylic surface after the prosthetic device is covered by the acquired saliva pellicle. Two main superficial characteristics are involved and facilitate the adhesion; these features are the internal roughness (1-3 μm) and surface free-energy values. When the pellicle is formed, hydrophilicity of *Candida albicans*

becomes an important adherence factor due to the increased surface wettability (PEREIRA-CENCI et al., 2007; HAHNEL et al., 2010; RODRIGUEZ ACOSTA et al., 2014).

Different treatment mechanisms have been developed through the years focusing on the direct effect of various substances against the peculiar fungus using topical therapy or parenteral drug administration (LAUDENBACH; EPSTEIN, 2009; EMAMI et al., 2014). Topical therapy includes chemical solutions such as chloride, glutaraldehyde and chlorhexidine (GAMA et al., 2015; RODRIGUEZ ACOSTA et al., 2015); physical methods such as photodynamic therapy, laser, ozone and microwaves (CAMPANHA et al., 2007; CARDOSO et al., 2008; LYON et al., 2013; ROSSONI et al., 2014; CARMELLO et al., 2015); different organic solutions: extracts of different plants such as *Equisetum giganteum* (MANSOURIAN et al., 2014; ALAVARCE et al., 2015); incorporation of chemical substances to hard or soft denture bases such as myconazole, silver nano particles and chlorhexidine (NEPPELENBROEK et al., 2008; ACOSTA-TORRES et al., 2012; BUENO et al., 2015). This whole universe of treatment options has successfully inhibited the studied microorganism but, on the other hand, some of these procedures might alter properties of the heat-polymerized acrylic resins (MIMA et al., 2008; PINTO LDE et al., 2010; SILVA et al., 2011; PARANHOS HDE et al., 2013).

These conventional mechanisms could produce a fungal resistance by gene mutations, especially when fungicides are applied (XIE et al.; COWEN et al.); in other words, they directly attack the cell membrane or metabolic functions. New approaches are looking for non-antibiotic/antifungal compounds capable of avoiding microbial resistance response and interact with human cells' surfaces (i.e., epithelial cells) (MCCARRON et al., 2004) or medical devices (i.e., catheters, acrylic resins) (ALI; ALHARBI; SURESH, 2013), altering the external physical properties exerting an anti-adherent effect (MINAGI et al., 1985).

The previous statement introduced the idea of developing a coating material in which the internal prosthetic surface would be modified only on its outer layer: roughness and surface free-energy values, without changing chemical or physical properties of the heat-polymerized acrylic resins (MINAGI et al., 1985; MCCARRON

et al., 2004; MCCARRON et al., 2007). Eventually, these coatings could be associated with a recognized anti-microbial agent and become a drug delivery system when exposed to the oral environment.

Therefore, cyanoacrylates had been used for that purpose in order to successfully prevent bacterial development with no effect on *Candida albicans* biofilms (BLUM; NOLTE; ROBERTSON, 1975; TSENG et al., 1990; GIRAY et al., 1993; DE ALMEIDA MANZANO et al., 2006). Due to their adhesives properties and bactericide effect, they are considered as tissue adhesives widely accepted in surgical procedures. But in the recent years, the fungus has been inhibited in two researches. One of them is a thesis of our team (TÁVORA, 2011) in which the inhibitory effect was achieved with ethyl-cyanoacrylate adhesive. Ali et al (ALI, 2013) have also reported inhibitory results using a different hydroxyl chain in the cyanoacrylate composition, the octyl-cyanoacrylate adhesive.

Organic adhesives are frequently used in surgeries in the recent years; especially fibrin sealants, which are recognized as a promising product capable of replacing conventional sutures. Fibrin sealant derived from snake venom was kindly supplied by the Center for the Study of Venoms and Venomous Animals (C.E.V.A.P.) of UNESP, its constituents and instructions for use are stated in its patents (registration numbers BR1020140114327 and BR1020140114360). At the time of use, the components were previously thawed, reconstituted, mixed and applied according to the protocol of the project (BARROS et al., 2009; GASPAROTTO et al., 2014; SEABRA FERREIRA, 2014).

Regarding these premises, it can be possible to apply a coating on the acrylic resin modifying its superficial properties (MCCARRON et al., 2007) as they inhibit the *Candida albicans* biofilm formation without altering its chemical or physical composition and dimensional stability (MINAGI et al., 1985). Cyanoacrylates with different hydroxyl chains and a Fibrin sealant were tested in order to compare inhibitory properties.

2 Article

The article presented in this Dissertation was written according to the *Gerodontology* instructions and guidelines for article submission.

TITLE PAGE:

Anti-Adherent and Antifungal Activity of Synthetic and Organic Adhesive Polymers on *Candida albicans* biofilms

RUNNING TITLE:

Inhibition of *Candida albicans* by adhesive polymers

KEYWORDS:

Candida albicans, Cyanoacrylates, Fibrin Sealant derived from Snake-venom, heat-polymerized acrylic resin.

Abstract

OBJECTIVE: To analyze the effect of synthetic and organic adhesive on *Candida albicans* biofilms.

BACKGROUND: Prevention and management of *Candida*-associated denture stomatitis have been widely studied but still looking for an irrefutable mechanism. In the last years, the innovative application of Cyanoacrylates and Fibrin Sealant derived from Snake-venom with antimicrobial purposes has caught attention in medical fields. These coating materials could act directly on the applied surface causing cytopathic or cytotoxic effects on cells and microorganisms.

MATERIALS AND METHODS: Fibrin sealant and cyanoacrylates (CA) with different hydroxyl chains were coated on one hundred seventy seven specimens (2-3 μ m Ra) and inoculated with *Candida albicans* SC5314 (1.10⁷ cells/mL) for 24 h. They were submitted to colony-forming units (CFU/mL) quantification assay, Colorimetric assay XTT and one specimen of each group was analyzed using Confocal Laser Scanning Microscopy for a qualitative analysis. The data was analyzed using Kruskal-Wallis and Dunn's multiple comparisons tests (p<0.05).

RESULTS: According to the CFU assay a growth reduction was observed in Octyl-CA (p<0,0000), Ethyl-CA Conventional (p<0,0002) and Ethyl-CA Gel (p<0,0002) groups with 87, 81 and 71 % respectively. The XTT assay demonstrated a decreased metabolic activity in Octyl-CA (70%) (p<0,0000) and Ethyl-CA conventional (61%) (p<0,0000). The Confocal Images showed a reduced fungal colonization in all CA groups with no sign of non-viable adhered cells on Octyl-CA.

CONCLUSIONS: Based on these findings, an anti-adherent effect against *Candida albicans* can be achieved by coating OCA and ECAC on heat-polymerized acrylic resin. Furthermore, more tests must be held on in order to understand antifungal effect.

Key words: *Candida albicans*, Cyanoacrylates, Fibrin Sealant derived from Snake-venom, adhesive polymers, heat-polymerized acrylic resin.

Introduction.

Candida-associated denture stomatitis is one of the most common oral mucosal diseases affecting all segments of the population (1), especially complete denture wearers, with greater incidence in elder and immune-compromised patients (2-5). *Candida* spp. are usually found in more than 90% of healthy individuals living in a symbiotic environment with other fungi and bacteria promoting homeostasis (3, 4).

In the oral cavity, an intimate contact between the denture and mucosa must be establish in order to provide an adequate adaptation and, consequently, great retention (6). The internal surface receives a gentle polishing incapable of turning into a smooth surface as the external aesthetic surface receives ($< 0.02 \mu\text{m Ra}$) (7). This different polishing seeks to preserve the close contact between the mucosa and complete denture but, at the same time, these high roughness values of internal surface of dentures create a pleasant reservoir for the fungi (8, 9).

Candida albicans (*C. albicans*) is the main microorganism isolated in the majority of prime fungal oral infections and represents more than 60% of biofilm structure (10, 11). In an unbalanced state, this opportunistic pathogen could colonize the prosthetic device followed by tissue invasion if the conditions were not restored to baseline (1-4). Its unique adherence faculty to the prosthesis and oral mucosa in its virulent state draws attention to researchers and clinicians, categorizing this pathology as *Candida*-associated denture stomatitis (1, 2).

The current treatments are focused on the inhibition of *C. albicans* (1, 12) and other related microorganisms (13-15) or reduce its adhesion to the internal prosthetic surface in acrylic base dentures (16). These contemporary methods include chemical

and physical interactions, as well as organic and synthetic products (12, 17-29). Inhibitory and sub inhibitory percentages against *C. albicans* have been obtained due to the product concentration and chemo-physical nature (30). Some fungicides compounds have induced mutations on *Candida* spp. providing a resistance capability to some drugs (31, 32), turning the scenario more complicated for future reinfections (33). But the effects on the prosthetic surfaces have also been evaluated, promoting some undesirable modifications such as higher roughness values, micro-hardness variations, limited residual effect and other well-documented side effects (34-38).

New approaches are focusing on replacing the well-known fungicides by non-antimicrobial agents capable of exerting an anti-adherent effect against specific microorganisms (39-43). Cells could be targeted (i.e. epithelial cells) with a nano-particle coating in order to interact with specific membrane receptors (44-49) or, on the other hand, target the medical device (i.e. catheters, denture bases) to prevent future colonization (42, 50-52).

The coating materials have been introduced in order to interact with the superficial characteristics of heat-polymerized acrylic resin (HPAR) such as roughness and surface free-energy values, which are considered as the main *Candida* adhesion mechanisms (9, 39, 51-54) and the different binding forces related to strong adherence capability (i.e. fungal adhesines, hydrophobic forces, cell zeta potential) (13, 48, 55, 56), or by its intrinsic drug release effect (50, 57). This new proposal has acclaimed greater advantages when compared to current disinfection procedures because of the fact that the resin composition would not be exposed and modify by neither physical nor chemical methods (58-60).

Initial tests showed unsatisfactory outcomes of different cyanoacrylates (CAs) when exposed to *C. albicans* biofilms, but they showed antibacterial effect against gram-positive microorganisms (61-65) and produced cytotoxic and cytopathic effects on human fibroblasts in different percentages (66-68). But it was also reported a stable prolonged residual effect of CAs on gingival fibroblast in a repaired prosthesis for 14 days when the adhesive was degraded (69). In the recent years, CAs have caught the researchers' interests by its application on different scenarios for example neurosurgery, colon surgery, periodontal surgery and *C. albicans* inhibition (50, 70-77).

Fibrin sealant derived from Snake-venom tissue adhesives have also entered in the surgical field successfully replacing conventional sutures, which could promote undesirable microbial colonization (64, 78-82). Fibrin sealant derived from snake venom produced by UNESP has not demonstrated bacteriostatic effect (64) and have been widely used in periodontal surgeries as well (81, 82).

Based on this review, coating materials could be considered as an ideal treatment because of its superficial effect without compromising the acrylic resin structure and properties (51, 52, 59). The aim of the study was to evaluate the fungal inhibitory effect of adhesive polymers, widely used in surgical procedures (75-77, 82, 83), when applied on resin surfaces. The null hypothesis is the cyanoacrylates adhesive polymers and Fibrin sealant derived from Snake-venom do not inhibit *Candida albicans* biofilms on HPAR. They were divided into two groups according to its nature: Synthetic nature, including cyanoacrylates

adhesives with different hydroxyl chains; and Organic nature: Fibrin sealant derived from Snake-venom.

Materials and Methods

Materials

Commercially available Cyanoacrylates were acquired with different hydroxyl chains: Ethyl Cyanoacrylate conventional (ECAc), Ethyl Cyanoacrylate Gel (ECAg), Butyl Cyanoacrylate (BCA) and Octyl Cyanoacrylate (OCA). Fibrin sealant derived from snake venom was kindly supplied by the Center for the Study of Venoms and Venomous Animals (C.E.V.A.P.) of UNESP.

Specimens

One hundred and seventy seven specimens were fabricated from a Zetalabor silicone pattern (Hard 85 Shore-A, Zhermack, Italy) (10 x 10 x 3 mm) using a plastic matrix, under 5 Kg for 10 minutes between two glass slabs previously isolated (84). These silicone patterns were flaked according to fabricant instructions into heat-polymerized acrylic resin (Vipicril Plus, VIPI Indústria, Comércio, Exportação e Importação de Produtos Odontológicos Ltda, Pirassununga, SP, Brazil) in water bath (Solab, Piracicaba, SP, Brazil). After polymerization, the specimens were trimmed using a metal bur (Maxi-cut; Dentsply-Maillefer, Ballaigues, Switzerland) and, one surface was polished for 15 seconds using a 120-grained sandpaper (Norton Abrasivos, São Paulo, Brazil) in a circular polisher (PLFDV, Fortel Indústria e Comércio, São Paulo, SP, Brazil) under refrigeration using a stainless steel circular device. The specimens were cleaned in an ultrasonic device (Ultrasonic Cleaner, Unique Indústria e Comércio, Indaiatuba, SP, Brazil) (9) for 20 minutes with distilled water in order to remove any contaminants and artifacts from acrylic surface. The surface roughness value was established between 2-3 μm in a roughness mobile

measuring station (Surftest SJ-301, Mitutoyo Corporation, Kanagawa, Japan) testing six different sites on each specimen (8). To eliminate residual monomer, the specimens were immersed in 2 mL of distilled water at 37 °C for 48 hours in a 24-well plate in an Oven for Bacteriological culture (Fanem, Sao Paulo, Brazil) according to ISO 1567:1988 (International Organization for Standardization Specification 1567, 1988). Finally, the specimens and brushes (Brush Tips, Plasdent Co., China) were sent to ethylene oxide sterilization center (ACECIL, Campinas, Brazil) (84, 85).

Coating Materials

The specimens were coated with each adhesive polymer receiving one drop at a time, approximately 50 µL, and uniformly distributed on the surface using a brush tip and excess was removed in the initial seconds. The coating was performed assuming that it would be thinner than the available gap between the gypsum casts and a complete denture, approximately 200 microns (86, 87) and its presence would not affect the denture adaptation. Initial tests revealed that the applied double coatings had a thickness between 30 and 60 microns. Additional information is shown in Table 1. (TABLE 1)

***C. albicans* standardization**

Initially, *C. albicans* (SC5314) were incubated in tryptic soy broth (TSB) (Accumedia Manufactures, Inc. Lansing, USA) added with 1% chloramphenicol (Quemacetina Succinato/ Carlo Erba®, Milano, MI, Italy) for 24 hours at 30°C in aerobic conditions (88). After this period, the fungal suspension was centrifuged during 10 minutes at 5000 rpm at 22°C for separation of microorganisms. The fungi were washed twice with Phosphate Buffered Solution (PBS) and the final pellet was resuspended in 1 mL of PBS. Therefore, 10 µL of the suspension were diluted in PBS in the proportion

of 1:10.000 and the fungi were counted on hemacytometer (89) to adjust the concentration. The fungal suspension was standardized in the concentration of 1×10^7 cells/mL (53).

Biofilm Formation on specimens

After ethylene oxide sterilization (A.C.E.C.I.L. Com. Ind. Ltda, Campinas, Brazil), all specimens were exposed to 2 mL of Artificial Saliva in order to develop the acquired pellicle for 2 hours at 37°C in a 24 wells plate (Cell Culture Plate, Nest Biotech Co., Ltd., China), and then they were dried at room temperature for 30 minutes (90). Then, they were introduced into a 24 wells plate with 1 mL of fungal suspension and remained in orbital shaking (75 rpm) for 90 minutes at 37°C (53, 91). They were washed twice in 1 mL of PBS in order to remove non-adhered cells (84) and they were immersed into 1 mL of Roswell Park Memorial Institute solution (RPMI-1640, Gibco®, GrandIsland, NY) and remained in orbital shaking (75 rpm) for 24 hours at 37°C (53, 89, 92). This procedure was adopted and standardized for all assays.

CFU/mL assay

Once the biofilm remained in RPMI for the experimental period, each specimen was washed in 1 ml of PBS and the biofilm was removed using a Sterile Cell Scraper 3010 (Costar, Sigma-Aldrich Co., Toluca, Mexico) into a 24 wells plate with 1 mL of PBS with constant force in all directions, regarding that the specimens should be submerge in the solution. Immediately, it was performed a serial dilution taking 100 μ L from the original plate and incorporated to a new well containing 900 μ L of PBS. This dilution was repeated three times until four dilutions were obtained ($10^{-1} - 10^{-4}$).

According to a pilot study, all dilutions were tested to obtain an adequate colony count for all experimental groups and the 10^{-2} dilution was constant in all groups. Once the dilutions were obtained in the plate, an aliquot of 25 μ L of the suspension was sown with sterile Drigalski strap in a petri plate containing BHI Agar (Agar Bacto BD and BBL™ Brain Heart Infusion, Difco Laboratories, Le Pont de Claix, France) with 5ug/mL of chloramphenicol. The petri plates were incubated at 37 °C for 24 hours. After this procedure, colonies were counted and introduced into the formula: $CFU/mL = \text{Number of colonies} \times \text{dilution}^{\text{factor}} / \text{serial dilution}$. Experiments were performed in triplicates.

XTT assay

The colorimetric assay XTT (2,3 Bis (2-Methoxy-4-Nitro-5-Sulfophenil) -5- [(Phenyl-Amino) Carbonyl] – 2H-Tetrazolium Hidroxide (Sigma Aldrich Inc., St Louis, MO, EUA) revealed the presence of metabolic activity which provided vital information about the fungi. It was performed after 24 hours of biofilm formation at 37 °C (93-95). The specimens were washed into PBS and submerged with 1 mL of XTT solution (1 mg/mL mixed with menadione in 1 mM concentration with PBS containing 200 mM glucose) (96) in the absence of light, remaining in orbital shaking at 75 rpm for 3 hours at 37 °C. Then, an aliquot of 1 mL was removed from each plate and transferred to a microtube (Safe-lock 2.0 mL, Eppendorf do Brazil Ltda., São Paulo, SP, Brazil). Once in the microtubes, they were centrifuged (10.000 rpm) at 10 °C for 2 minutes in order to obtain cellular decantation in Centrifuge (Centrifuge 5417R, Eppendorf, Hamburg, Germany). An aliquot of 200 μ L of supernatant was transferred to a 96 wells plate in triplicate (Cell Culture Plate, Nest Biotech Co., Ltd., China). The absorbance was measured using the multiplate reader at 550 nm (Synergy Mx

Monochromator-Based, Biotek, Washington, DC, USA). Experiments were performed in triplicates.

Confocal Laser Scanning Microscopy

After 24 hours of incubation in RPMI, the specimens were washed in 1 mL of PBS and then submersed in 1 mL LIVE/DEAD® BacLight™ L7007 solution (Molecular Probes, Invitrogen Brazil Ltd., Sao Paulo, SP, Brazil) (84) for 20 minutes in a 24 wells plate in the absence of light at room temperature. The fluorescence was determined by two flurochromes which would stain the cell's membrane: SYTO®-9 for viable cells (green) and Propidium Iodete for dead cells (red) (84). When the time elapsed, they were located with the selected face turned down on a glass slide and analyzed using a Confocal Microscope Leica TCS-SPE (Leica Mycrosystems, Mannheim, Germany), from which digital images were obtained.

Statistical Analysis

The tests employed were Kruskal-Wallis and Dunn's multiple comparisons tests ($p < 0.05$).

Results

According to the CFU assay at 24 hours, the shortest and largest hydroxyl chain CA exhibited a high inhibitory result: OCA (87%), ECAC (81%) and ECAG (71%). BCA showed the SV clearly facilitated *Candida* growth (106%). These values are shown in Figure 1, 2 and Table 2.

FIGURE 1 AND 2

TABLE 2

In the XTT assay at 24 hours, OCA demonstrated the highest value among experimental coatings with 70%, followed by ECAC with 61% and ECAG with 40%. The BCA group showed a slightly metabolic alteration (35%) and the SV group enhanced the *Candida*'s metabolism (-4%). These results are shown in Table 2.

According to statistical analysis, CG showed significant difference when compared to ECAG, ECAC and OCA; while SV presented difference with all CA groups. Among CA groups, BCA and OCA were statistically different. This data relies on Figure 3.

FIGURE 3

Confocal Images at 24 hours of biofilm development revealed that all CA groups have reduced fungal colonization and showed viable and non-viable cells adhered to the surface. Except in OCA group, low count of viable cells were observed but an absence of non-viable adhered cells were noted. ECAC group showed a low account but some non-viable adhered cells were observed. Surfaces coated with SV allowed a fungal development as good as CG (97%).

FIGURE 4

Discussion.

This study revealed that CAs coatings could decrease *C. albicans* adhesion on HPAR. The null hypothesis was rejected for tested CAs. On the other hand, SV favored fungal development with no metabolic alterations.

In initial studies, antifungal effects were not reported using CAs (62, 63, 66, 97) or SV (64, 98). The present study showed an antifungal activity with CAs could be related to a possible modification on its composition or inability to establish strong binding interactions (48). Other factor such as the influence of release products <formaldehyde and cyanoacetate compounds> (62, 69) could be also considered because CAs tend to decrease its cytopathic effect on cells within 14 days and remain with low reactions for 28 days (70, 72, 73). Since a prolonged degradation of CAs occurs, *C. albicans* could adapt or colonize the surface when the coatings were completely or partially degraded.

Based on literature, it was expected that a short hydroxyl chain CA could exert higher microbial or cellular alteration (62, 69) and a bigger chain would not be as cytopathic or cytotoxic as the previous ones (70, 73). ECAc, ECAg and BCA behaved as expected (64) because as the hydroxyl chain gets bigger, the cellular alteration would be diminished. However, according to the chain-related release product rate, OCA showed a contradictory result exerting the highest values.

On the CFU assay, OCA and ECAc showed the highest values among CAs with considerable reduction of fungal growth on HPAR, 87% and 81% respectively. However, ECAg and BCA could achieve a reduction above 60%. These findings could be explained as a high release rate sub-product of ECAc exerting a cytopathic

effect on fungal growth (62), a reduction of rough values (9), modification of zeta cell value (55) or the presence of a physical barrier that could prevent fungal approximation of 10 nm in order to establish irreversible attachment (48, 55).

The CFU assay provides limited data and requires complementary tests for better interpretation (99); those tests could be a metabolic assay or a staining technique. The metabolic assay (XTT) did not report a complete antifungal activity. The fungal respiratory metabolism followed a similar pattern as seen in CFU. The OCA and ECAC exerted higher metabolic alterations, 70 and 61% respectively. ECAG and BCA showed a discrete functional modification. The staining technique using Live/Dead assay kit on CLSM showed compatible images with low fungal adherence on OCA and ECAC coatings. In OCA image, the low fungal count was restricted to viable cells with compatible images when sodium hypochlorite was used (27, 84). On the other CAs, non-viable adhered cells were also found. This outcome could be explained as a possible release of sub-product due to a degradation process by time and their diffusion in moist environments (62, 67, 100). These tests could not identify the exact mechanism but certainly it could support future researches trying to explain the *Candida*'s interaction.

The only published data with antifungal effect of CAs belongs to Ali et al. (50). They concluded that OCA could completely prevent fungal adhesion after eleven days of biofilm colonization using proliferative assays based on counting cells/colonies and showed an antifungal activity of 100%. Even though, their methodology is refuted according to protocols established in literature (9, 53, 84), the present study demonstrated an anti-adherent property of 87% at 24 h, respiratory alteration of 60% and CLSM images showed low count of viable adhered cells.

According to the present study, SV did not show any antifungal property. Similar data was published against different bacteria in different scenarios (64, 80, 81, 101-103). However, SV could resist in moist environments as a drug delivery system (57, 83) by the incorporation of antifungal substances for a sustainable effect and local prevention and management of *Candida*-associated denture stomatitis.

Our results demonstrated the fungistatic effect of different CAs but superficial characteristics, release products, fibroblast and epithelial responses and prolonged evaluation periods must be evaluated in future studies.

Conclusions

According to these results, CA clearly modified metabolic mechanisms of *C. albicans* (XTT) and exerted an anti-adherent effect (CFU/mL).

- OCA and ECAC showed superior antifungal effect in the performed tests among CAs:
OCA>ECAC>ECAG>BCA
- SV did not alter Candida's development or adhesion.

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Table I. Experimental groups and their respective commercial name, working time and nature. Working times were calculated according a pilot study. After each coating, all specimens remained 30 minutes for full polymerization.

Experimental groups	Product name	Working time	Nature
Control Group	VIPI Cril ®	None	None
Ethyl Cyanoacrylate Conventional	Superbond® Original	30 seconds	Synthetic
Ethyl Cyanoacrylate Gel	Superbond® Gel	40 seconds	Synthetic
Butyl Cyanoacrylate	Tekbond ® 932	4 minutes	Synthetic
Octyl Cyanoacrylate	Dermabond ® High Viscosity	15 seconds	Synthetic
Snake Venom	C.E.V.A.P.	2 minutes	Organic

Table II. CFU/mL with Median values and Interquartile Range of fungal count. The XTT assay showed a metabolic alteration in all CA groups with higher values in OCA and ECAC groups. P-values are shown for CFU and XTT assays.

<u>Groups</u>	<u>24 h Incubation Period</u>		
	<u>CFU/mL (10³)</u>		<u>XTT</u>
	<u>Count</u>	<u>P value</u>	<u>P value</u>
Ethyl Cyanoacrylate Conventional	110x10 ³ ± 184x10 ³	p<0,0002	p<0,0000
Ethyl Cyanoacrylate Gel	198x10 ³ ± 56x10 ³	p<0,0002	p<0,0004
Butyl Cyanoacrylate	276x10 ³ ± 420x10 ³	p = 7163	p<0,02
Octyl Cyanoacrylate	92x10 ³ ± 244x10 ³	p<0,0000	p<0,0000
Snake Venom	728x10 ³ ± 96x10 ³	1	1
Control Group	688x10 ³ ± 288x10 ³	--	--

Figure 1. CFU assay based on Median values. All percentages were compared to CG. At 24 hours, CAs showed a reduction on *Candida* growth above 60%. The higher values were obtained by OCA and ECAC, 87% and 84% respectively. SV enhanced fungal growth by 106%.

Figure 2. CFU assay at 24 hours and Median Values were used. ECAC, ECAG and OCA showed statistical difference against CG and SV. In CAs groups, OCA and BCA were statistical different. CG, SV and BCA did not show statistical difference. Kruskal-Wallis and Dunn's multiple comparison tests ($p < 0.05$).

Figure 3. Colorimetric assay XTT and its absorbance values. GC, SV and BCA had no statistical difference, while ECAC, ECAG and OCA were different from CG and SV. Among Cas, OCA and BCA showed significant difference. Analyzed by Kruskal Wallis and Dunn's multiple comparison tests ($p < 0.05$).

Figure 4. Confocal Images using the LIVE/DEAD assay kit. The experimental groups are ordered by: A) ECAC, B) ECAG, C) BCA, D) OCA, E) SV, and F) CG. At 24 hours, obtained images showed that CAs decreased fungal adherence on the coated surface (Green cells= viable; Red cells= non-viable). OCA only showed a reduced count of viable adhered cells, while ECAC showed low count of adhered cells. SV and CG demonstrated a considerable amount of adhered fungal cells.

3 Discussion

3 DISCUSSION

This research showed a clear difference with initial-published data about cyanoacrylates' effect on *C. albicans* inhibition (BLUM; NOLTE; ROBERTSON, 1975; EIFERMAN; SNYDER, 1983). The results confirmed that CAs did not show a fungicide effect but it could prevent colonization of *C. albicans* on HPAR and for this reason the null hypothesis was rejected for CAs groups. Comparable results were obtained in a published paper using a specific hydroxyl chain in CA composition (ALI; ALHARBI; SURESH, 2013).

Previous works have determined that formaldehyde and cyanoacetate compounds were released on fibroblast medium (Dulbelcco's Modified Eagle Medium, DMEM) due to a degradation process of CAs (THUMWANIT; KEDJARUNE, 1999) and they maintained a continuous effect on fibroblasts for at least two weeks (THUMWANIT; KEDJARUNE, 1999; LANDEGREN et al., 2010). This fact provides a firmly justification to keep testing CAs in prolonged periods to prevent or manage *Candida*-associated denture stomatitis.

These findings generated a doubt, why CAs could not prevent fungal adhesion or development in the past (BLUM; NOLTE; ROBERTSON, 1975): perhaps new formulations have been conceived in order to enhance adhesive properties as they, without meant it, modulated fungal colonization. Even though materials have evolved from commercially available and sterile presentation, literature is lack of updated evidence when testing four different CAs against this opportunistic pathogen.

In medical fields, CAs have achieved considerably inhibition of certain bacteria, mainly gram positive 70% (DE ALMEIDA MANZANO et al., 2006; CHEN et al., 2007). A bacteriostatic effect in those papers clearly draws attention how could these products achieve antimicrobial effects. The answers rely on exothermic reaction; high average release products and early exposure of partially polymerize CAs (TSENG et al., 1990; THUMWANIT; KEDJARUNE, 1999). However, human cells do not show a permanent damage and it was reported a transient effect of CAs

when applied on osteoblasts, epithelial cells and peripheral nerves (LANDEGREN et al., 2010; PARAL et al., 2011; DE MELO et al., 2013; ESTEVES et al., 2014).

Candida albicans could endure this transient effect and, when the coatings were degraded, colonize prosthetic surfaces. The present results showed a reduction of anti-adherence properties in some CAs at 24 hours evaluation period (View table 2). It is not clear if the CAs would overcome prolonged exposure in oral moist environment but available data on surgical fields showed a sustainable effect for 14 and 28 days and have been catalogued as non-toxic products (LANDEGREN et al., 2010; GRIMALDI et al., 2015; VIDA et al., 2015).

The only published data (ALI; ALHARBI; SURESH, 2013) using CAs had different methodologies starting with the biofilm colonization to removal of fungi from the prosthetic surface. The results acclaimed complete anti-adherent effect against *C. albicans* (100%) using OCA (2-Octyl Cyanoacrylate, Johnson & Johnson, Ethicon) and Adper Single Bond Adhesive™ (3M) could reduce considerably *Candida*'s growth after 11 days of incubation. It was not defined how the coatings were applied and other details such as paint technique, sterilization, working and setting times were missing. Our developmental protocol of biofilms is well documented in literature (PEREIRA-CENCI et al., 2007; DA SILVA et al., 2008; HAHNEL et al., 2010; DA SILVA et al., 2011) and the evaluation at 24 hours is essential to understand what is happening to the *Candida*'s adherence capability. Also, CFU/mL, the gold standard test to analyze cell's growth, needs complementary tests to interpret these results. The limitation of CFU relies on the colony count procedure because it is not considered the individual cellular response. Metabolic assays such as XTT could provide this data and how the respiratory function, carried out by the mitochondria, was affected. CLSM images provides valuable information if the fungus was adhered and died in the mid-time. This semi-quantitative test could not be measured by CFU or XTT assays and could help to extrapolate a release product effect carried out by previously reported degradation (TÁVORA, 2011; PANTANELLA et al., 2013; BUENO et al., 2015). As shown in CLSM images, fungal cells did not approximate to the acrylic surface and remained adhered on the coating.

However, short hydroxyl chains in CAs claimed to be more toxic than long and more biocompatible chains (THUMWANIT; KEDJARUNE, 1999), in this research

CAs did not behaved as expected. The difference was observed in a consistent fungistatic activity of OCA in all assays and a variable reduction on anti-adherent properties of other CA groups.

A possible theory could explain this different behavior. OCA was the only tested material that was sterile and had rapid polymerization working and setting time. For these reasons the whole OCA constituents would have remained intact in a full-polymerized coating capable of guarantee its integrity in moist environments. On the other hand, commercially available CAs (ECAC, ECAG and BCA) were acquired as a non-sterile presentation with extended working and setting times when compared to OCA.

Fibrin sealant derived from Snake Venom could not exert an antifungal effect. The null hypothesis was accepted with this group. Many works have determined its biocompatible nature on stem cells (GASPAROTTO et al., 2014) as well for periodontal surgery (BARBOSA; GREGH; PASSANEZI, 2007; BARBOSA et al., 2008). However, SV has an ineffective application with antimicrobial functions (CHEN et al., 2007). The reasons to explain these findings (View Table 2, Figure 1-2 and Image 1) could be associated to the nature of this adhesive. Biological adhesives could act like a metabolic source for *C. albicans*, which could make this coating a pleasant nutrient reservoir since they are biodegradable, bio-absorbent, non-toxic and non-immunogenic (SEABRA FERREIRA, 2014). It might explain the high metabolic response as shown in XTT assay and in 24 hours CFU assay, and its growth was better than CG.

It's known that SV have a low resistance when tension forces are applied (CHEN et al., 2007). But since they do not have antimicrobial function, studies had combined with some antibiotics (MARONE et al., 1999; PATRLJ et al., 2000; FARHAT et al., 2007). This new approach must be tested against *C. albicans* since the fabrication process, cost-effect relationship and ease application could turn it into an effective drug deliver system (Seabra Ferreira, 2014).

Many hypotheses could be listed based on the present results on CAs (View Table 2, Figure 4 and Image 1). One possible explanation could be a modification on the superficial properties, because one of the objectives of coating materials is to modify surface roughness turning into a smooth surface decreasing fungal entrapment and retention. Also the hydrophobicity and cell zeta values could indeed hinder well-known fungal adherence mechanisms mediated by proteins and electrostatic interactions.

Other theory could be the presence of the coating as a physical barrier, keeping away the fungus from the substrate (MCCARRON et al., 2004; MCCARRON et al., 2007). If the irreversible distance could not be reached, *C. albicans* would not adhere by its strong interactions forces. The fact that CAs with short hydroxyl chains, ECA and BCA, could initially release more sub-product compounds (TSENG et al., 1990; THUMWANIT; KEDJARUNE, 1999), it could lead to rapid reduction of the coating thickness and an exposure of HPAR surface promoting the adhesion of *C. albicans*.

It can be suggested to perform CFU, XTT and CLSM assays with new periods to understand the time progression of CAs: 12 and 48 hours, 7 and 14 days (SILVA et al., 2012; BUENO et al., 2015). These new intervals could bring important details to interpret initial, late mature and prolonged effect of the anti-adherent mechanisms against *C. albicans*.

In order to look for answer to this anti-adherent mechanism, an interesting assay could be performed trying to identify the CAs sub-products. We know which substances were released in the past (THUMWANIT; KEDJARUNE, 1999; LANDEGREN et al., 2010), but it was demonstrated that something on its composition has changed. This idea could be the beginning for a possible isolation of non-drug products with antifungal activity reducing the chance for resistance mutations of *Candida spp.*

Other important tests such as ROS assay, MTT assay, Contact angle, Free-energy surface values and Stability against chemical disinfection are being developed by our team with interesting preliminary results.

4 Conclusion

4 CONCLUSION

According to the present study it can be concluded:

- All CAs could prevent Candida's adherence on HPAR
 - OCA and ECAC showed the higher metabolic and anti-adherent values at 24 hours
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6Annex

Annex 1. Inhibitory Halo formed by Sodium Hypochlorite 1% (A), SV (B) and SV mixed with Chlorhexidine (C).