

EVALUATION OF GUANIDINE ANTIFUNGAL SOLUTIONS FOR DENTURE BASE RESIN: AN *IN VITRO* STUDY

Avaliação de soluções antifúngicas de guanidina para resina base de dentadura: um estudo *in vitro*

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RESUMO

Antecedentes: Os objetivos deste estudo foram: (1) formular soluções de cloridrato de polihexametileno guanidina (PHMGH) em diferentes concentrações; (2) avaliar sua atividade antifúngica contra biofilme maduro de *Candida albicans* em resinas acrílicas; (3) avaliar efeitos na resistência à flexão e rugosidade da superfície. **Métodos:** Soluções de PHMGH foram formuladas com água destilada e 0,125, 0,250 ou 0,5% em peso de PHMGH. Um grupo sem PHMGH foi usado como controle. Para análise da atividade antifúngica, amostras de resina acrílica foram contaminadas com *C. albicans*, e imersas em soluções de PHMGH ou água destilada por 5 ou 10 min. Resistência à flexão final e rugosidade da superfície foram avaliadas. **Resultados:** Todas as soluções de PHMGH aos 5 ou 10 minutos apresentaram atividade antifúngica em comparação ao grupo controle ($p < 0,05$). O grupo com 0,5% em peso de PHMGH não mostrou colônias contáveis, independentemente do tempo. Após 10 minutos, todas as soluções de PHMGH tiveram efeito antifúngico sem diferenças de 0,125 a 0,5% em peso de PHMGH. Os grupos apresentaram alta resistência à flexão após o contato com as soluções compatíveis com a recomendação ISO 20795-1: 2013. Os valores de rugosidade da superfície permaneceram baixos, de 0,01 a 0,04 μm para todos os grupos. **Conclusão:** Dentro das limitações deste estudo, concluiu-se que o uso de uma solução composta por água destilada e 0,5% em massa de PHMGH por 5 min foi eficaz como agente desinfetante contra o biofilme maduro de *C. albicans*, mantendo rugosidade e resistência à flexão aceitáveis. **Palavras-chave:** Resinas acrílicas. Antifúngicos. Candidíase. Guanidina. Dentaduras.

ABSTRACT

Background: The purposes of this study were: (1) to formulate polyhexamethylene guanidine hydrochloride (PHMGH) solutions at different concentrations; (2) to evaluate their antifungal activity against a mature biofilm of *Candida albicans* on acrylic resins; (3) to evaluate possible effects on acrylic resins flexural strength and surface roughness. **Methods:** PHMGH solutions were formulated with distilled water and 0.125, 0.250, or 0.5 wt.% of PHMGH. One group without PHMGH was used as control. For antifungal activity analysis, acrylic resin specimens were contaminated with *C. albicans*. Specimens were immersed in PHMGH solutions or distilled water for 5 or 10 min. Ultimate flexural strength and surface roughness of acrylic resins were evaluated. **Results:** All PHMGH solutions at 5 or 10 min showed antifungal activity compared to the control group ($p < 0.05$). The group with 0.5 wt.% of PHMGH showed no countable colonies regardless of the time of contact with the acrylic resins. After 10 min, all PHMGH solutions had antifungal effect without differences from 0.125 to 0.5 wt.% of PHMGH. All groups showed high flexural strength after contact with the solutions compatible with ISO 20795-1:2013 recommendation. The values of surface roughness always remained low, from 0.01 to 0.04 μm for all groups. **Conclusion:** Within the limitations of this study, it was concluded that the use of a solution composed by distilled water and 0.5 wt.% of PHMGH for 5 min was effective as a disinfectant agent against mature biofilm of *C. albicans*, maintaining acceptable roughness and flexural strength. **Keywords:** Acrylic resins. Antifungal agents. Candidiasis. Guanidine. Dentures.

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INTRODUCTION

Denture associated stomatitis is a high-prevalence disease related to the *Candida* presence (60 to 100% of those who wear dentures), deficient oral and denture hygiene, high roughness of resin, and trauma¹. *Candida albicans*, the most widespread specie of the genus *Candida* in oral stomatitis^{1,2}, is observed in 81.4% of denture users with stomatitis³. Moreover, this is one of the most common microorganisms related to nosocomial pneumonia (NP) and ventilator-associated pneumonia (VAP), increasing the costs to health care annually⁴. Oral and denture hygiene could assist in reducing the risk for NP and VAP and spare health care resources⁵⁻⁷.

Initial adhesion of *Candida* to denture base resin is facilitated due to the retentive areas of polymers^{8,9}. The mechanical removal of microorganisms from acrylic resin is a challenge^{2,9}. In this context, classical disinfectants, as citric acid, enzymatic agents¹⁰, sodium hypochlorite (NaOCl), glutaraldehyde, chlorhexidine, and sodium perborate, are chemical agents used¹¹. However, they may decrease resins microhardness, flexural strength, and increase the surface roughness¹²⁻¹⁴. Commercial mouthwashes such as those disinfectants composed by alcohols, sodium hydroxide, and sodium benzoate, and those without alcohol but composed by chlorhexidine digluconate, cetylpyridinium chloride, phosphoric acid, and sodium benzoate were also previously tested in acrylic resins and negatively affected microhardness and surface roughness^{15,16}. In this context, disinfectant solutions that do not compromise physical properties and surface texture of acrylic resins are necessary to reduce the biofilm formation¹⁷. In this way, general users of dentures and patients with higher susceptibility to suffering from candidiasis, such as those in intensive treatment in hospitals, could have the benefits of lower microbial colonization in the prosthetic device.

Polyhexamethylene guanidine hydrochloride (PHMGH) is an effective antimicrobial polymer against bacteria, virus¹⁸ and fungi¹⁹. This molecule has a high solubility in water, and the benefit of been colorless and odorless²⁰⁻²². In dentistry, PHMGH was added into different blends to formulate restorative materials such as dental sealants²³ and resin infiltrants²⁴. In both studies, the resins with PHMGH showed antibacterial activity against other important microorganisms involved in an oral disease (caries): *Streptococcus mutans*. More recently, PHMGH was tested in aqueous solution for disinfecting denture liners, and it reduced the biofilm of *C. albicans* on the material without changing its tensile strength²⁵. However, acrylic resins and liners differ between them. Liners are composed of acrylic resins and plastifiers or silicone elastomers, while the resin for dentures is basically composed of polymethylmethacrylate (PMMA). The composition of these materials leads to distinct behavior and, consequently, different clinical indications. The surface texture of liners, with increased porosity in comparison to PMMA, along with their higher solubility, makes them more prone to microbial adhesion and loss of properties^{26,27}. Therefore, liners are usually used as temporary materials to cover the base of dentures, reshaping them, and decreasing the impact of masticatory forces on the oral mucosa²⁸. In other hand, dentures are intended to present a long time of use, supporting the masticatory forces and providing a complex oral rehabilitation²⁶. The search for strategies to improve the longevity of prosthodontic rehabilitation and decrease the susceptibility of *Candida* colonization on PMMA is important to maintain the serviceability of dentures and postpone their replacement, besides keeping a healthy oral environment.

The purposes of this study were: (1) to formulate PHMGH aqueous solutions at different concentrations; (2) to evaluate their antifungal activity against a mature biofilm of *C. albicans* on acrylic resins; (3) to evaluate possible effects on acrylic resins flexural strength and surface roughness.

METHODS

Figure 1 summarizes the study design. Briefly, the solutions were prepared with different concentrations of PHMGH. Then, the samples were prepared for the microbiological assay or physical tests. For the physical tests, the samples were analyzed for roughness, immersed in the solutions, and then tested for ultimate flexural strength and roughness again (in this sequence). The samples were exposed to the PHMGH solutions for 5 or 10 min to be tested, and the evaluations were performed once for each test.

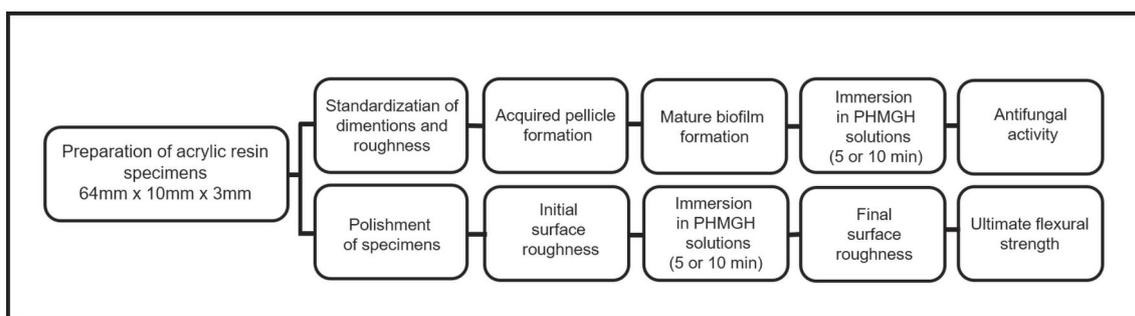


Figure 1: Flowchart of the study design.

PHMGH solutions formulation

Polyhexamethylene guanidine hydrochloride (PHMGH, Figure 2) was purchased (Haihang Industry, Jinan City, China) was used without further purification. PHMGH was weighted using an analytical balance (AUW220D, Shimadzu, Kyoto, Japan) with sterile instruments. PHMGH at 0.125 wt.%, 0.250 wt.%, or 0.5 wt.% was mixed with sterile distilled water under aseptic condition, and one group remained without PHMGH as control. Therefore, four groups were formulated and tested: control group (G^{CTRL}), $PHMGH^{0.125\%}$, $PHMGH^{0.25\%}$, and $PHMGH^{0.5\%}$.

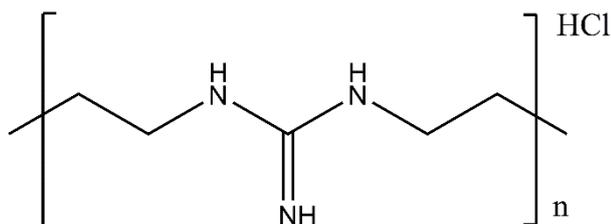


Figure 2: The chemical structure of polyhexamethylene guanidine hydrochloride (PHMGH) used in the study to formulate the antifungal solutions for denture base resin.

Preparation of acrylic resin specimens for antifungal activity evaluation

The acrylic resin specimens (64 mm long, 10 mm wide, 3 mm thickness) were prepared using a standard dental metal flask for heat polymerization. The powder and the liquid of acrylic resin (Artigos Odontológicos Clássico, São Paulo, Brazil) were mixed at 2:1 by weight. The dental flasks were pressed with 500 kg, opened, and the excess of acrylic resin was removed. The dental flasks were pressed again with 1000 kg and immersed in water in a polymerization unit after 30 min at 75 °C for 8 h. The flasks were allowed to bench cool for 4 h.

The specimens were removed, cut in squares (30 mm²), and ground flat (400, 600, and 1200-grit) under running water.

The surface roughness of the specimens was measured using a rugosimeter SJ-201 (Surface Roughness Tester, Mitutoyo, Japan). The mean of three courses of 0.25 µm on each side was evaluated and standardized in 0.3-0.4 µm²⁹. There were no special randomization procedures performed. The samples were standardized for roughness, placed inside a container, and they were drawn one by one from the container to be divided into eight groups.

Reactivation process and growth conditions

Candida albicans (ATCC 10231, INCQS 40006, Fundação Oswaldo Cruz, batch number 101440006b) frozen in skin milk was plated (50 µL) on Sabouraud Dextrose agar (Aldrich Chemical Co., Missouri, USA) in Petri dish. The dishes were kept at 37 °C for 24 h in an incubator at aerobic conditions. After this period, the colonies of *Candida albicans* grown on the agar were collected to be used in the following assay. For this purpose, a bacteriological loop was filled twice with the microorganism on the Petri dish and immersed in 25 mL of BHI with 0.5 wt.% of glucose for 24 h at 37 °C. After 24 h, 100 µL of this solution was collected, serially diluted in Eppendorf tubes with saline solution, and plated on Sabouraud Dextrose agar in Petri dishes to obtain the value of inoculum to be used in the following assay, which corresponded to 1.5 x 10⁷ cells/mL. All incubation periods were performed under aerobic conditions²⁹.

Acquired pellicle formation

A healthy volunteer (25 years old), who had not used antibiotics, mouth rinses, or any other medication that could affect the saliva composition and flow in the past three months signed an informed consent approved by the local Ethics Committee (2.780.491) to participate in the study providing stimulated saliva. The collected saliva passed through 0.22 µm-filter (Millipore Corporation, Massachusetts, USA) with phosphate-buffered saline (PBS) solution (KCl at 50 mM, KPO⁴ at 1 mM, CaCl² at 1 mM, MgCl² at 0.1 mM, pH 6.5) at 1:1. The specimens were submitted to hydrogen peroxide plasma sterilization and immersed in 1 mL of saliva for 30 min at 37 °C²⁹.

Adherence of *C. albicans*

The specimens were removed from the tubes, transferred to sterile centrifuge tubes with 1 mL of the previously prepared suspension of *C. albicans*, and incubated at 37 °C for 2 h (to yeast adherence phase). For biofilm maturation, the specimens were gently washed with saline solution (0.9% NaCl) and transferred to a new centrifuge tube containing BHI broth and 0.5 wt.% of glucose for 72 h at 37 °C aerobically. The medium was renewed every 24 h to form 72 h-mature biofilms on specimens²⁹.

Antifungal activity of PHMGH solutions in mature biofilms

The infected specimens were dipped at PHMGH solutions for 5 min (n=3) or 10 min (n=3). G^{CTRL} remained without PHMGH solution contact. This group was only immersed in distilled water. The specimens were washed with 5 mL of saline solution, transferred to sterilized centrifuge tubes, and vortexed during 1 min in 1 mL of saline solution with sterile glass beads to allow the biofilm disruption. The solutions were diluted until the 10⁻⁶ mL. Two drops (25 µL each) of each dilution were plated on Sabouraud Dextrose agar in Petri

dishes. The dishes were aerobically incubated for 36 h at 37 °C to count the colonies visually. The results were expressed in colony-forming units per milliliter after logarithmic transformation (\log CFU/mL)²⁵.

Specimens preparation for ultimate flexural strength and surface roughness

The specimens were prepared according to 2.2 section³⁰ and polished (Model 3v, Arotec, São Paulo, Brazil). First, silicon carbide sandpapers were used with 600, 1200, and 2000-grit. Then, the specimens were finally polished with a felt disc and a suspension of aluminum oxide of 0.5 μ m.

Ultimate flexural strength

ISO 20795-1:2013 was used to analyze the possible effects of PHMGH-aqueous solutions on the flexural strength. Therefore, five specimens per group were used according to ISO recommendation³⁰. The specimens were immersed in the PHMGH-aqueous solutions and immediately tested for flexural strength using a universal testing machine (EZ-SX Series, Shimadzu, Japan) at 5 mm/min. Three-point bending tests were carried out with 50 (\pm 0.01) mm between the supporting points. Ultimate flexural strength was calculated according to ISO 20795-1:2013³⁰.

Surface roughness

The surface roughness of specimens was analyzed before and after the immersion in the PHMGH-aqueous solutions. For this purpose, the specimens were analyzed at five random sites on the surface using the rugosimeter SJ-201. The device provided the mean of three measures (0.5 mm/s, 0.25 μ m) per analysis. The results were displayed using the parameter that shows the arithmetical mean height (Ra) in μ m.

Statistical analysis

SigmaPlot, version 12.0 (Systat Software, Inc., San Jose, CA, USA) was used to analyze the data. Shapiro-Wilk test was applied to evaluate the data distribution. Data on antifungal activity evaluation were analyzed via two-way ANOVA and Tukey post hoc tests. The ultimate flexural strength was analyzed in accordance with ISO 20795-1:2013. Paired t-tests were applied within the same group to compare the values of surface roughness before and after the immersion in PHMGH-aqueous solutions. A level of significance of 0.05 was considered for all tests.

RESULTS

The results of antifungal activity are displayed in Figure 3. Two-way ANOVA indicated interaction for time and concentration ($p=0.007$). When the acrylic resins with a mature biofilm of *C. albicans* were exposed for 5 min to the aqueous solutions of PHMGH, there was decreased biofilm viability. After 5 min of contact with the solutions, the biofilm values decreased from 4.85 (\pm 0.10) \log CFU/mL for control group containing just distilled water to 00.00 (\pm 0.00) \log CFU/mL for the group exposed to 0.5 wt.% of PHMGH solution ($p<0.05$).

When exposed for 10 min, the antifungal activity also occurred using PHMGH aqueous solutions ($p<0.05$). PHMGH^{0.125%} and PHMGH^{0.250%} showed increased antifungal activity after 10 min in contact in comparison to 5 min ($p<0.05$). Moreover, there was no difference between 0.125, 0.250, and 0.5 wt.% of PHMGH solutions after 10 min of action ($p>0.05$).

The solution with the highest concentration of PHMGH showed no colonies able to be count on the Petri dishes regardless of the time of contact (5 or 10 min). In this way, this group (PHMGH^{0.5%}) showed no statistically significant differences between 5 and 10 min of action ($p > 0.05$, Figure 3).

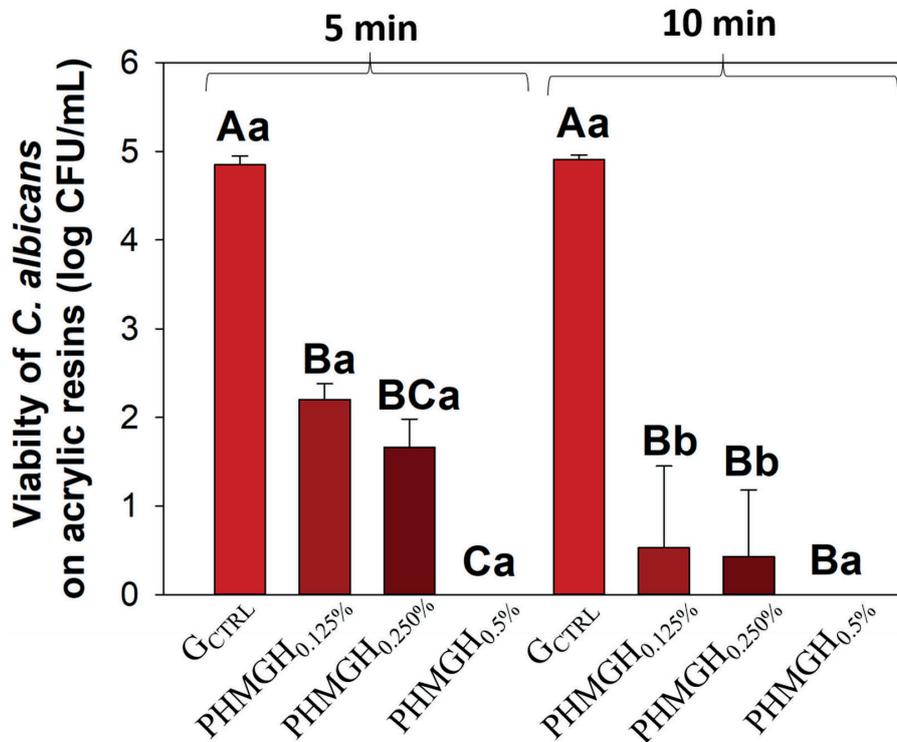


Figure 3: Results of antifungal activity of the solutions tested against mature biofilm of *C. albicans* on acrylic resins. The results are expressed in mean and standard deviation values of colony-forming units per milliliter (CFU/mL) after logarithmic transformation according to different concentrations of PHMGH and immersion times. Different capital letters indicate a statistically significant difference among groups in the same period of contact (5 or 10 min) ($p < 0.05$). Different lowercase letters indicate a statistically significant difference between the same group comparing different periods of contact (5 or 10 min) ($p < 0.05$).

The results of ultimate flexural strength are expressed in mean and standard deviation values in MPa in Figure 4. The results ranged from 77.8 (± 7.2) to 90.1 (± 8.7) MPa. ISO establishes that denture base polymers Type 1 (heat-polymerizable materials), Class I (powder and liquid), should present not less than 65 MPa of ultimate flexural strength. Therefore, all polymers achieved values following ISO recommendation³⁰.

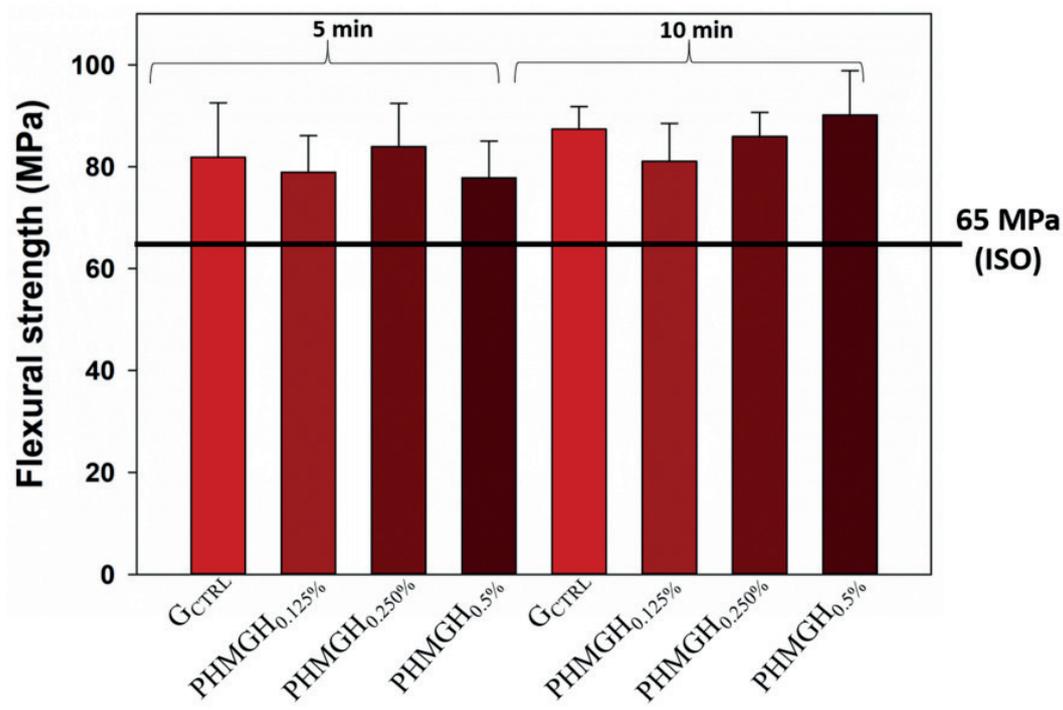


Figure 4: Results of the ultimate flexural strength of acrylic resins after the immersion in PHMGH solutions. The results are expressed in MPa, and they are compared with ISO values.

The surface roughness of acrylic resins ranged from 0.01 (± 0.00) to 0.04 (± 0.01) in both periods: before or after the contact with the solutions (Table 1). There were no statistical differences among groups regardless of the PHMGH concentration and period of immersion (5 or 10 min) ($p > 0.05$).

Table 1: Mean and standard deviation values of initial and final surface roughness according to different concentrations of PHMGH and immersion times.

Groups	Immersion Time	Initial Surface Roughness (Ra)	Final Surface Roughness (Ra)
G _{CTRL}	5 min	0.02 (± 0.00) ^a	0.02 (± 0.02) ^a
PHMGH _{0.125%}		0.02 (± 0.01) ^a	0.01 (± 0.00) ^a
PHMGH _{0.25%}		0.02 (± 0.01) ^a	0.02 (± 0.00) ^a
PHMGH _{0.5%}		0.01 (± 0.00) ^a	0.02 (± 0.01) ^a
G _{CTRL}	10 min	0.03 (± 0.00) ^a	0.04 (± 0.01) ^a
PHMGH _{0.125%}		0.04 (± 0.01) ^a	0.03 (± 0.01) ^a
PHMGH _{0.25%}		0.03 (± 0.00) ^a	0.04 (± 0.01) ^a
PHMGH _{0.5%}		0.02 (± 0.00) ^a	0.02 (± 0.00) ^a

The same letters indicate no statistically significant difference in the same column ($p > 0.05$).

DISCUSSION

In the present study, PHMGH solutions were formulated to disinfect denture base resin after the formation of mature biofilm of *C. albicans* on their surfaces. All PHMGH concentrations from 0.125 to 0.5 wt.% showed antifungal activity. Moreover, the flexural strength and the surface roughness of the denture resins were not affected, showing that the solutions formulated may be an interesting strategy to disinfect denture resins. The high solubility in water associated with PHMGH colorless and odorless assisted in PHMGH solutions formulation^{20,21}. Further, the non-corrosive property of PHMGH may provide benefits to the partial removable prosthesis with a metallic structure^{20,21}.

PHMGH presents a broad-spectrum activity against Gram-positive and Gram-negative bacteria and previously showed effectiveness at lower concentrations and at shorter times²⁰. A previous study formulated a PHMGH solution with distilled water, and the results showed that the concentration of 0.52% in 90 s of contact and 0.36% in 3 min of contact eliminated *Bacillus subtilis* spores in a stain steel or glass surface²¹. PHMGH antimicrobial mechanism is based on electrostatic interaction with constituents with negative charges from cell wall and membrane, leading to cellular disruption, leakage of intracellular constituents, and cytosol coagulation²⁰. Here we observed that the higher the PHMGH concentration, the greater the antifungal effect achieved. Also, for the highest concentration group (PHMGH^{0.5%}) with 5 or 10 minutes of immersion time, there was a total elimination of viable fungal colonies to be counted. However, even in the lowest concentration and time immersion used (PHMGH^{0.125%}), the antifungal efficacy was achieved, reducing more than 2 logs compared to G^{CTRL}.

It is worth to mention that after 10 min of contact between the mature biofilms and the PHMGH solutions, there was no difference among PHMGH^{0.125%}, PHMGH^{0.250%}, and PHMGH^{0.5%} ($p > 0.05$). In other words, the lowest concentration of PHMGH (0.125%) could be used instead of the highest (0.5%) in a situation with 10 min of contact. Based on the present study, the disinfection for 5 min with the solution containing 0.5 wt.% of PHMGH is a promising strategy. This concentration showed no statistical difference for 0.250 wt.% of PHMGH for 5 min but showed a higher percentage decrease in fungal viability in comparison to 0.250 wt.% and no difference comparing the 5 min and 10 min-values. Therefore, it seems that a shorter contact time could be used (5 min instead of 10 min) with the highest concentration. This result may have a positive impact when the cytotoxicity concern is addressed. Despite the high antibacterial activity, when previously used in humidifier disinfectants, it was suggested that molecules of polyhexamethylene guanidine led to toxicological effects³¹. In this way, the possibility of use PHMGH for a short time of contact with the material may bring advantages, decreasing possible PHMGH remnants on polymer structure and cytotoxic effect. Even though the toxic events were observed for patients that had inhaled polyhexamethylene guanidine for long periods, which differs from the use of PHMGH here proposed, more tests should be performed to evaluate possible cytotoxicity of PHMGH solutions before their application to analyze if the material is liable to be handled by the patients without presenting any health risk.

An important topic to be addressed is that based on the findings of this study, it is not possible to conclude whether there is an interaction between PHMGH and other agents used for disinfection (such as surfactants), as well as between PHMGH and organic residues present in the acrylic resin. These interactions could reduce the antifungal activity of the solutions. Moreover, in an *in vivo* scenario, greater biodiversity, and biofilm complexity would be involved, which affects the virulence of each microorganism and its response to antimicrobial agents³²⁻³⁴. Thus, the analysis of the solutions against mature and multi-specie biofilms should be further investigated. It is possible that *in vivo* scenario, the complexity of biofilm leads to the necessity of mechanical disinfection, and the use of PHMGH solutions as an adjuvant in the process.

During disinfection procedures, acrylic resins are susceptible to interact with chemical solutions and suffer surface and structural modifications, decreasing the flexural strength and increasing the surface roughness¹²⁻¹⁴. Among commonly used disinfectants, NaOCl performs better against *C. albicans*, however, it modifies the material structure¹¹, as slight color change¹³, and increases in surface roughness. An ideal disinfectant solution should not decrease denture acrylic resin properties. In this study, PHGMH solutions did not change the flexural strength of acrylic resins regardless of the PHMGH concentration and the time of evaluation, with values always higher in comparison to ISO cut-off at 65 MPa³⁰.

Immersion in disinfectant solutions may increase surface roughness^{15,16}, increasing microorganisms' adhesion, biofilm formation, and associated diseases^{1,5,7}. The increase of surface roughness leads to the further difficulty of biofilm removal, as it adheres to the rough surface and penetrates through porosities, facilitating the survival of microorganisms against regular hygiene methods²⁷. Disinfectant solutions of NaOCl and glutaraldehyde previously indicated changes in resin roughness^{8,35}. The surface roughness maintenance was a positive effect of formulated solutions. As shown in Table 1, PHMGH solutions at different concentrations did not modify the surface of the heat polymerized acrylic resins after immersion regardless of the time of evaluation. The paired t-tests between before and after immersion values for each group showed a power of analysis lower than 80%. However, even if the increase in the number of samples led to a statistically significant difference, the differences in roughness micrometers would be well below the values commonly established in the literature as important for microbial adhesion. Generally, the threshold value for microbial adhesion in the oral environment is accepted as 0.2 μm ⁹, and more recently 0.1 μm ³⁶. In the present study, the highest values were at 0.04 μm , being very lowest than these cut-offs. It is a limitation of this study not to have tested for more extended periods of immersion. Future studies with more cycles of disinfection are required to simulate long-term use and address with should be the frequency of PHMGH solutions use. This assessment could also answer if the colorless of the solutions, qualitatively observed by the authors during PHMGH solutions formulation, would be beneficial to not modify color parameters and visual clinical aspects of the disinfected dentures. Moreover, other surface parameters, such as those to measure the maximum profile valley depth (Rv), total profile height (Rt), and maximum height of profile (Rz) could be further investigated to add more information about the topographic feature.

In the present study, promising antimicrobial solutions were formulated with a molecule from guanidine family, PHMGH. This material was tested against acrylic resin for dental use the first time. The solutions showed activity against a mature biofilm of *C. albicans*, without changing the physical properties of the materials.

CONCLUSION

Within the limitations of this study, it was concluded that the use of a solution composed by distilled water and 0.5 wt.% of PHMGH for 5 min was effective as a disinfectant agent against mature biofilm of *C. albicans*, maintaining acceptable roughness and flexural strength.

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