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Efeito da solução de nanopartículas de quitosana no biofilme de *Candida* spp. e nas propriedades de superfície da resina acrílica

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Com vocês compartilho esta conquista e toda minha gratidão.

"Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível. "

Charles Chaplin

RESUMO

Introdução: A propriedade antifúngica da quitosana possibilita sua aplicação como inibidor da formação de biofilme sobre próteses dentárias. **Objetivos:** Sintetizar, caracterizar e avaliar a atividade antifúngica da solução de nanopartículas de quitosana (ChNP) sobre *Candida* spp., na aderência, formação e redução do biofilme maduro, bem como nas propriedades físicas da resina acrílica e na redução do biofilme de *Candida* spp. desenvolvido na superfície da mesma. **Metodologia:** A solução de 3,8 mg/mL nanopartículas de quitosana (ChNP) foi obtida pela técnica de geleificação iônica. A caracterização da solução foi realizada por espectroscopia de absorção na região do infravermelho (FTIR) e microscopia eletrônica de transmissão (MET). Foi realizado a determinação da Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima (CFM) frente as cepas de *C. albicans* ATCC 60193, *C. albicans* ATCC 10,231, *C. albicans* CBS 562, *C. tropicallis* CBS 94 e *C. krusei* CBS 73 utilizando como controle positivo o hipoclorito de sódio 1% (NaOCl). A análise da cinética de crescimento por contagem de unidades formadoras de colônias (UFC) sobre *C. albicans* ATCC 60193 e a alteração da micromorfologia fúngica frente a diferentes concentrações da ChNP e hipoclorito de sódio foram avaliadas. A inibição da aderência inicial, formação e redução do biofilme maduro de *C. albicans* foi realizado em microplacas de 96 poços e com exposição das soluções por 1 min e 8 h, a % de inibição das células fúngicas foi obtida utilizando cristal de violeta e leitura das placas por meio de leitor de microplacas, com comprimento de onda de 600 nm. O controle positivo foi a solução de hipoclorito de sódio 1% e o controle negativo foi a solução de cloreto de sódio. Para a análise de aderência e colonização de *Candida spp.* sobre a base de resina acrílica foram confeccionados quarenta e oito corpos de prova (2 x 4mm) de resina acrílica, os quais foram distribuídos em quatro grupos (n=6): G1(controle), G2 (ChNP CIM), G3 (ChNP CIM x 4) e G4 (hipoclorito de sódio 1%). Os testes foram realizados em placas de 24 poços, e as soluções permaneceram em contato por 8h diárias após biofilme multi-espécie de *C. albicans* ATCC 60193, *C. tropicallis* CBS 94 *e C. krusei* CBS 73 desenvolvido por 24 h. Os resultados foram avaliados em 48 h e 5 dias após o início do teste. A análise foi realizada por meio de contagem de UFC das cepas de *Candida* e comparação entre os grupos e a UFC inicial. Aferições de rugosidade (Ra) e microdureza (VHN) foram realizadas 24h antes e após o experimento. Os ensaios foram conduzidos em triplicata. Os dados foram estatisticamente e descritivamente analisados (α=0.05). **Resultados:** A $CIM_{83\%}$ encontrada da ChNP foi de 30,08 µg/mL, e a relação CIM/CFM demonstrou atividade fungicida para todas as cepas testadas. ChNP demonstrou inibição do crescimento fúngico quando comparado ao controle de crescimento e uma total inibição da CIM x 4 na cinética de crescimento, e mostrou inibição de pseudohifas e clamidoconídeos, apresentando somente a micromorfologia de blastoconídeos. Os resultados dos efeitos inibitórios do ChNP na adesão inicial, na formação e na redução do biofilme de *C. albicans* mostrou que a maior concentração testada (CIM x 4) foi a mais eficaz. A ChNP demostrou resultado positivo sobre a aderência inicial de *C. albicans*, uma vez que na CIM x 4 apresentou % I semelhante ao NaOCl ($p>0,05$). Depois de 1 min de contato, apenas ChNP em CIM x 4 apresentou maior % I na formação de biofilme (51,1%) do que NaOCl. Após 8 h em todas as concentrações, a ChNP mostrou maior I% (50,2; 56.6 e 57,8%) quando comparado ao NaOCl. Durante 72 h de incubação com 3 aplicações de 1 mim, ChNP foi capaz de inibir o biofilme maduro de *C. albicans* em 52,2% e reduziu o crescimento fúngico em 66,1%, após aplicar por 8 h. Além disso, ChNP com um aplicativo de 8 h tiveram %I maior do que o NaOCl. Apenas a concentração de MIC x 4 para a ChNP foi eficaz de inibir o biofilme maduro de 96 h, (6 x 1 min ou 2 x 8 h). Houve redução significativa de UFC de *Candida* spp. sobre a superfície de resina acrílica, após 48h para os grupos ChNP CIM x 4) e NaOCl Para o período de 5 dias, ChNP CIM x 4 apresentou redução de UFC ($2,0 \times 10^5$ UFC/mL) quando comparada ao

controle de crescimento (7,5 x 10^5 UFC/mL), entretanto, o NaOCl apresentou a maior redução UFC (3,0 x 10^4 UFC/mL) quando comparado as outras soluções. Com relação a alteração de superfície, apenas NaOCl apresentou aumento de Ra significante no período de 5 dias, enquanto todos os grupos apresentaram diminuição significante nos valores VHN, sendo a maior redução VNN para o grupo NaOCl. **Conclusão e** b**enefícios esperados:** A solução de nanopartículas de quitosana apresentou atividade antifúngica no biofilme maduro de *C. albicans* e atividade anti-biofilme de *Candida* spp. sobre resina acrílica, causando mínimas alterações de superfície no material, apresentando assim elevado potencial de uso como agente de desinfecção para próteses dentárias.

Descritores: Quitosana, *Candida*, Biofilmes, Prótese Dentária, Nanopartículas

ABSTRACT

Introduction: The antifungal properties of chitosan allows your application to inhibit biofilm formation on dentures. **Objectives:** to synthesize, to characterize and to evaluate the antifungal activity of chitosan nanoparticle solution (ChNP) against *Candida* spp.; on and this solution on initial adherence, formation and reduction of mature biofilm and the effect on surface properties on acrylic resin and the reduction of multi-species *Candida* spp. biofilm developed on this surface. **Methodology:** A solution of 3.8 mg/mL of ChNP was obtained by ionic gelation technique. The characterization of the solution was carried out by absorption spectroscopy in the infrared (FTIR) and transmission electron microscopy (TEM). The determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) was made against *C. albicans* ATCC 60193, *C. albicans* ATCC 10231, *C. albicans* CBS 562, *C. tropicallis* CBS 94 and *C. krusei* CBS 73 using as the positive control 1% sodium hypochlorite (NaOCl). The analysis of time-kill assays by counting colony forming units (CFU) of *C. albicans* ATCC 60193 and fungal micromorphology of the different concentrations of the ChNP and sodium hypochlorite were evaluated. The inhibition of initial adherence and reduced formation of mature biofilm of *C. albicans* was performed in 96-well plate and exposing to the solution for 1 min and 8 h, the % inhibition of fungal cells was obtained using crystal violet and reading plates using microplate reader with a wavelength of 600 nm. The positive control was 1% sodium hypochlorite solution and sodium chloride was the negative control. For the analysis of adherence and colonization of *Candida spp.* on acrylic resin base they were made forty-eight specimens (2 x 4mm) acrylic resin, which were divided into four groups (n=6): G1 (control), G2 (ChNP CIM), G3 (ChNP MIC x 4) and G4: 1% sodium hypochlorite. Tests were performed in 24-well plates, and the solutions remained in contact daily for 8 hours after multi-species biofilm of *C. albicans* ATCC 60193, *C. tropicallis* CBS 94 *C. krusei* CBS 73 developed for 24 hr. The results were assessed at 48 h and 5 days after beginning the test. Analysis was performed by counting the CFU *Candida* strains and compared between the groups and the initial CFU. Roughness measurements (Ra) and microhardness (VHN) were performed 24 hours before and after the experiment. The tests were conducted in triplicate. Data were statistically analyzed ($\alpha = 0.05$). **Results:** The $MIC_{83\%}$ found to ChNP was 30.08 g /mL, and the ratio CIM/CFM showed fungicidal activity for all strains tested. ChNP showed inhibition of fungal growth compared to fungal growth and complete inhibition at MIC x 4 at time-kill assays, and showed inhibition of pseudohyphae and clamidoconidios, presenting only the morphology of blastoconidia. The results of the inhibitory effects of ChNP in the initial adherence, formation and the reduction of *C. albicans* biofilm showed that the highest concentration (MIC x 4) was the most effective. The ChNP showed positive results on the initial *C. albicans* adherence, since ChNP at MIC x 4 had % I similar to NaOCl ($p > 0.05$). After 1 min contact time only ChNP at MIC x 4 showed higher % I on biofilm formation (51.1%) than NaOCl. After 8 h all concentrations of ChNP showed superior % I (50.2 to 57.8%) compared to NaOCl. During 72 h *C. albicans* mature biofilm ChNP was effective to inhibit 52.2 to 66.1% of fungal growth after 3 applications of 1 min or 1 application of 8 h, regardless of its concentration. Moreover, ChNP with 1 application of 8 h had superior % I than NaOCl. However, for 96 h *C. albicans* biofilm, only ChNP at MIC x 4 concentration was effective to inhibit mature biofilm (6 x 1 min or 2 x 8 h). There was a significant reduction in CFU *Candida* spp. on the surface of acrylic resin, after 48 hours for groups ChNP MIC x 4 and NaOCl. For the period of 5 days, ChNP MIC x 4 decreased CFU (2.0 x 105 CFU / mL) as compared to the growth control (7.5 x 105 CFU / mL), however, NaOCl showed the greatest reduction in CFU (3.0 x 104 CFU / mL) when compared to other

solutions. Regarding the surface change, only NaOCl showed a significant increase in Ra in 5-day period, while all groups had a significant decrease in VHN values, with the largest reduction VHN to the NaOCl group. **Conclusion and expected benefits:** The solution of chitosan nanoparticles showed antifungal activity in mature biofilms of *C. albicans* and anti-biofilm activity of *Candida* spp. on acrylic resin, causing minimal surface changes in the material, thus presenting high potential for use as a disinfection agent for dental prostheses.

Keywords: Chitosan, *Candida*, Biofilms, Dental Prosthesis, Nanoparticles

SUMÁRIO

1. INTRODUÇÃO

Candida albicans é encontrada como um micro-organismo comensal no trato digestivo de mamíferos (NADĂŞ et al., 2013), assim como na cavidade oral dos seres humanos (PEREIRA et al., 2013). É também o fungo patogênico mais comum nos seres humanos, causando infecções das mucosas, especialmente em indivíduos imunocomprometidos (PFALLER; DIEKEMA, 2007). Vários fatores podem predispor os indivíduos a candidíase, como o tratamento prolongado com antibióticos e corticosteróides, presença de diabetes mellitus, deficiências nutricionais, doenças imunossupressoras ou terapia hormonal (MAYER; WILSON; HUBE, 2013).

O crescimento microbiano sobre a superfície de próteses dentárias resulta da adesão de células microbianas reforçadas pela rugosidade da superfície da prótese e por interações adesivas entre as espécies de *Candida*, principalmente *C. albicans*, e bactérias orais (ZAMPERINI et al., 2010). Eles podem induzir uma resposta inflamatória crônica na mucosa oral, descrito como estomatite protética, que se caracteriza por ser a doença infecciosa mais comum que afeta a mucosa oral e é altamente prevalente em indivíduos portadores de prótese dentária (MOSKONA; KAPLAN, 1992).

Nesse contexto, o tratamento das lesões de estomatite protética envolve a orientação da higienização das próteses e da cavidade bucal, a remoção de um possível fator irritante (próteses dentárias mal adaptadas e insatisfatórias), o uso de antifúngicos e a confecção de uma nova prótese. Os métodos mecânicos e químicos são frequentemente aconselhados para a higiene das próteses dentárias para evitar colonização de micro-organismos (PARANHOS et al., 2014; MARRA et al., 2012; YILDIRIM-BICER et al., 2014), já que *C. albicans* está presente em 11% a 67% dos usuários de próteses totais devido à falta de higiene (ARENDORF e WALKER, 1987).

Assim, várias soluções antissépticas com a finalidade de auxiliar na higiene de próteses dentárias vem sendo relatadas na literatura (PARANHOS et al., 2014; MARRA et al., 2012; YILDIRIM-BICER et al., 2014; SATO et al., 2013; PESCI-BARDON et al., 2006). Para este fim, estas soluções devem apresentar como principais características não possuírem toxicidade, serem facilmente removidas e não deixarem resíduos que possam ser irritantes, não danificar os materiais utilizados na fabricação da prótese, serem estáveis no armazenamento e possuírem atividade bactericida e fungicida (PESCI-BARDON et al., 2006). Estas características nem sempre são obtidas em todos os componentes das soluções,

tornando o desenvolvimento de novos agentes desinfetantes uma busca na comunidade científica.

A quitina é um biopolímero linear de unidades de N-acetilglicosamina ligadas por união glicosídicas ß-(1-4) presente nos exoesqueletos da maioria dos crustáceos, sendo considerado o segundo maior biopolímero disponível na natureza e sua produção é estimada em 109-1011 toneladas ao ano (DASH et al., 2011). A parcial desacetilação alcalina da quitina resulta na quitosana, a qual é um polissacarídeo composto de unidades de glicosamina (2-amino-2-deoxi-D-glicopiranose) e N-acetilglicosamina (2-acetamido-2-deoxi-D-glicopiranose) ligados por união glicosídica ß-(1-4) (KONG et al., 2010). A quitosana tem muitas propriedades biológicas interessantes, incluindo biocompatibilidade e biodegradabilidade em produtos não tóxicos e fisiologicamente inertes, como também atividades hemostática, fungiostática, antibacteriana e antitumoral, o que leva este biopolímero a possuir aplicação em diversas áreas do conhecimento, como: biotecnologia, agricultura, cosmética, médica, farmacêutica, alimentícia, (KITTUR et al. 2005) embalagens de alimentos e indústria têxtil (COOKSEY, 2005; PHAECHAMUD, 2008; RABEA, 2003).

A quitosana pode ser sintetizada através da desacetilação da quitina em meio alcalino. O processo de conversão de quitina em quitosana deve ser realizado de forma adequada para garantir a produção de quitosana com alta qualidade e pureza, livre de contaminantes, como proteínas, endotoxinas e metais tóxicos. O polímero obtido deve ser caracterizado de acordo com o grau de desacetilação e massa molar ou peso molecular, uma vez que tais características podem influenciar na degradação e hidrólise dos polissacarídeos (COSTA SILVA; SANTOS; FERREIRA, 2006; PEDRO et al., 2009). Além disso, a quitosana é um biopolímero solúvel em meio ácido que apresenta caráter catiônico devido aos grupos amino (NH_2), os quais em baixo pH são protonados em amínio (NH^{3+}) e podem interagir com componentes carregados negativamente, como proteínas, polissacarídeos aniônicos e fosfolipídeos (COSTA et al., 2014; PHAECHAMUD, 2008).

Uma das propriedades mais significantes da quitosana é a atividade antimicrobiana contra vários microrganismos, incluindo algas, fungos e bactérias (RABEA et al., 2003; COSTA et al., 2014; SILVA-DIAS et al., 2014). Além disso, estudos demonstram que a atividade antifúngica da quitosana é influenciada pelo peso molecular, grau de desacetilação, concentração do polímero, fonte de coleta da quitosana e os tipos de grupos funcionais presentes nas cadeias de derivados de quitosana (ZIANI et al., 2009; GUO et al., 2006).

Basicamente, a atividade antifúngica da quitosana é atribuída pela natureza policatiônica da quitosana, exibindo atividade antifúngica natural, sem a necessidade de qualquer modificação química (ZIANI, et al., 2009). Existem três mecanismos propostos como o modo de atuação da quitosana em fungos. No primeiro mecanismo, a membrana plasmática dos fungos é o alvo principal da quitosana. A carga positiva do biopolímero permite interagir com os componentes dos fosfolipídios carregados negativamente da membrana dos fungos, diminuindo a carga negativa na superfície das células (para valores mais positivos). Esta mudança diminui a concentração efetiva dos cátions na superfície, principalmente de K+, o que altera o equilíbrio do estado estacionário entre a concentração interna/externa da membrana plasmática e a diferença de potencial elétrico, aumentando assim a permeabilidade da membrana. Este aumento provoca perda do conteúdo celular, que subsequentemente conduz à morte das células (GARCÍA-RINCÓN et al., 2010; LIU et al., 2004). Para o segundo mecanismo, a quitosana age como um agente quelante por ligação a oligo-elementos, tornando indisponíveis os nutrientes essenciais para o crescimento normal dos fungos (ROLLER; COVILL, 1999). Por último, o terceiro mecanismo indica que a quitosana pode penetrar na parede celular dos fungos e se ligar ao seu DNA. Isto irá inibir a síntese de RNAm e, assim, afetará a produção de proteínas e enzimas essenciais (KONG et al., 2010).

A quitosana têm mostrado atividade antifúngica (PEÑA et al., 2013), principalmente contra *C. albicans*, porém esta atividade tem sido bastante avaliada em diversos compostos a base de quitosana, como oligoquitosana (KULIKOV et al., 2014), quitosana em hidrogel (SILVA DIAS et al., 2014), quitosana associado ao nitrato de cerio (COBRADO et al. 2013) e solução de nanopartículas de quitosana (ING et al., 2012). Esta diversidade na formulação de agentes a base quitosana interfere na atividade antimicrobiana e dificulta a comparação entre os estudos.

Peña, Sánchez e Calahorra em 2013 investigaram o mecanismo de ação da quitosana de baixo peso molecular nas leveduras patogênicas de *C. albicans*, foi observada diminuição no crescimento do fungo e foi concluído que, devido aos fatores que agem na atividade fungicida da quitosana, concentrações superiores a 1,0 mg/mL devem ser utilizadas. Acima desta concentração uma ação fungicida pode ser assegurada, e não apenas fungiostática. Porém, poucos estudos avaliaram a atividade da quitosana na formação e redução do biofilme de espécies de *Candida*. Silva-Dias et al. (2014) investigaram a atividade do hidrogel de quitosana de baixo peso molecular em biofilme de *Candida* spp. em placas de 96 poços, e foi demonstrado que a quitosana reduziu consideravelmente a atividade

metabólica do biofilme (até 90%), tanto no biofilme inicial como na desagregação da biomassa do biofilme maduro.

As nanopartículas de quitosana possuem um grande potencial para liberação de agentes terapêuticos na cavidade bucal, como drogas, vacinas, genes, proteínas e peptídeos (NEILANDS et al., 2011), e podem ser formadas pelo processo de geleificação iônica. Este processo promove a formação de nanopartículas por meio de interações eletrostáticas entre as cadeias de quitosana carregadas positivamente e poliânions utilizados como agentes de reticulação, como o tripolifosfato de sódio (TPP). O TPP tem sido bastante utilizado porque não é tóxico, possui rápida capacidade de geleificação e interage eletrostaticamente com a quitosana catiônica em um método rápido e simples (AYDIN; PULAT, 2012; ING et al., 2012).

Ing et al. em 2012 investigaram a atividade antifúngica da quitosana em sua forma natural e em solução de nanopartículas de quitosana contra *Candida albicans, Fusarium solani* e *Aspergillus niger*. A solução de nanopartículas de quitosana em diferentes concentrações de baixo e alto peso molecular apresentaram maior atividade antifúngica contra *C. albicans*, com concentração inibitória mínima menor (0,25 – 1,0 mg/mL) quando comparada com a quitosana em solução natural (3 mg/mL). O melhor efeito inibitório da solução de nanopartículas de quitosana foi devido ao tamanho da partícula sintetizada. Entretanto, este estudo foi conduzido em células planctônicas e a literatura ainda é escassa com investigações sobre o efeito de soluções de nanopartículas de quitosana na aderência, formação e redução do biofilme de *C. albicans.*

A patogenicidade e resistência às drogas dos fungos imobilizados no biofilme bucal, enfatiza a necessidade do desenvolvimento de novos agentes antimicrobianos que possam inibir a formação de biofilme ou reduzir as células do biofilme maduro sem afetar os tecidos bucais e as propriedades do material no qual o biofilme é formado. Porém, o potencial uso da solução de nanopartículas de quitosana como agente de desinfecção de base de prótese ainda não foi investigado.

Assim, o objetivo deste trabalho foi sintetizar e caracterizar uma solução de nanopartículas de quitosana, avaliar sua atividade anti-fúngica na aderência, formação e redução do biofilme de *C. albicans*, bem como investigar o efeito desta solução na inibição do biofilme multi-espécie de *Candida* spp. formados na superfície da resina acrílica de base de próteses, como também nas propriedades de rugosidade e microdureza superficial deste material.

2. OBJETIVOS

Objetivo geral:

Sintetizar e caracterizar uma solução de nanopartículas de quitosana e avaliar a atividade antifúngica sobre *Candida* spp. em estado planctônico e biofilme formados na superfície da resina acrílica termopolimerizável, como também nas propriedades de rugosidade e microdureza superficial deste material.

Objetivos específicos:

- Sintetizar e caracterizar a solução de nanopartículas de quitosana pelos métodos de espectroscopia de absorção na região do infravermelho (FTIR) e microscopia eletrônica de transmissão (MET);
- Verificar a concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) da solução de nanopartícula de quitosana sobre cepas de *C. albicans* ATCC 60193, *C. albicans* ATCC 10231, *C. albicans* CBS 562, *C. tropicallis* CBS 94 e *C. krusei* CBS 73*;*
- Avaliar o efeito da solução de nanopartículas de quitosana na CIM e CIM x 4 na aderência, formação e redução do biofilme de *C. albicans* ATCC 60193 pelo método de pigmentação com cristal de violeta;
- Avaliar o efeito da solução de nanopartículas de quitosana com menor CIM na inibição da formação do biofilme multi-espécie de *C. albicans, C. tropicallis* e *C. krusei* sobre a superfície da resina acrílica termopolimerizável por 48h e 5 dias, bem como nas propriedades de superfície.

3. CAPÍTULO 1

Neste capítulo o trabalho será descrito em formato de artigo, o qual será submetido para publicação no periódico *The Journal of Prosthetic Dentistry*.

Effects of chitosan nanoparticles solution on inhibition of *Candida* multi-species biofilm formed on acrylic resin surface and in its physical properties

Abstract

Objectives: This study aimed to synthesize, characterize and evaluate the antifungal activity of chitosan nanoparticle solution (ChNP) against *Candida* spp. The effect of ChNP was evaluated on initial adherence, formation and reduction of mature biofilm of *C. albicans* and on surface properties on acrylic resin and the reduction of multi-species *Candida* spp. biofilm developed on this surface. **Methodology:** The characterization of ChNP was carried out by absorption spectroscopy in the infrared (FTIR) and Transmission Electron Microscopy (TEM). The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) was made against *Candida albicans*, *Candida tropicallis* and *Candida krusei* using as positive control 1% sodium hypochlorite (NaOCl). The analysis of time-kill assays and changes on fungal micromorphology of the different concentrations of ChNP and NaOCl were also evaluated. The % of inhibition (%I) on initial adherence, biofilm formation and reduction of mature biofilm of *C. albicans* were evaluated by exposition of ChNP aand NaOCl at CIM, CIM x 2, CIM x 4 concentrations for 1 min and 8 h with different frequencies of application during 24h or 48h mature biofilm. For the antifungal effect of solutions on *Candida* spp. biofilm formation on acrylic resin surface, 48 specimens of acrylic resin were prepared. The solutions remained in contact daily for 8 h after 48 h and 5 days *Candida* spp. multi-species biofilm developed. Counting of *Candida* cells (CFU), Roughness (Ra) and Vickers microhardness (VHN) surface measurements on acrylic specimens were performed before and after biofilm. Data were analyzed statisticaly and descriptively ($\alpha = 0.05$). **Results:** The $MIC_{83\%}$ found to ChNP was 30.08 mg /mL, and the ratio CIM/CFM showed fungicidal activity for all strains tested. ChNP show complete inhibition at MIC x 4 at time-kill assays and and showed inhibition of pseudohyphae and clamidoconidios, presenting only the morphology of blastoconidia. The ChNP at MIC x 4 on the initial *C. albicans* adherence had % I similar to NaOCl. After 1 min contact time only ChNP at MIC x 4 showed higher % I than NaOC and after 8 h all concentrations of ChNP showed superior % I compared to NaOCl. During 72 h *C. albicans* mature biofilm ChNP was effective to inhibit fungal growth after 3 applications of 1 min or 1 application of 8 h. However, for 96 h *C. albicans* biofilm, only ChNP at MIC x 4 was effective to inhibit mature biofilm (6 x 1 min or 2 x 8 h). There was a significant reduction in CFU *Candida* spp. on the surface of acrylic resin, after 48 h, for groups ChNP MIC x 4 and NaOCl. After 5 days biofilm, ChNP at MIC x 4 decreased CFU, however NaOCl showed higher CFU reduction. After 5 days biofilm, only NaOCl showed a significant increase in Ra values, but all solutions had significant decreasing of VHN values. **Conclusions:** ChNP solution showed antifungal activity against mature biofilms of *C. albicans* and anti-biofilm activity of *Candida* spp. on acrylic resin, causing minimal changes on its surface.

Key-words: Chitosan, *Candida*, Biofilms, Dental Prosthesis, Nanoparticles

1. INTRODUCTION

Candida albicans is the most widespread opportunistic pathogenic yeast that lives commensally within the human body, but other clinical relevant *Candida* species are also found, as *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei*[8]. These microbial communities are the main cause of denture stomatitis that is a very common inflammation of oral mucosa in denture wearers. Denture stomatitis can be caused due to an association of factors such as mucosal trauma due to poor denture fit; smoking; immune deficiency; increasing age of the denture user; increased age of dentures and poor denture hygiene[9,10].

Mechanical and chemical methods are frequently advised for denture hygiene to avoid *Candida* spp. biofilm accumulation and to prevent oral mucosal inflammation and denture stomatitis[11]. Dentures can be cleaned mechanically, chemically, or by both methods[11,12]. The denture disinfection has been recommended as an essential procedure for preventing cross-contamination and the maintenance of a healthy oral mucosa[13].

A significant feature of the chemical method is the variety of possible active agents against fungal cells[12]. The most commonly chemicals agents used are enzymes, sodium hypochlorite and peroxide solutions[11,12] . However, the indiscriminate use of antifungal agents can result in the selection of resistant strains[14,15]. This fact justifies the development of new antifungal agents for use in daily clinical practice and for denture hygiene.

Chitin is the primary structural component of the shells of crustaceans, arthropods and the fungal cell wall. The partial deacetylation of chitin leads to chitosan, a polysaccharide composed of units of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine (2-acetamido-2-deoxy-D–glucose) linked by β(1→4) bonds. Due to its properties of biodegradability, biocompatibility, low toxicity and antimicrobial activity, chitosan has several biomedical applications[1].

The chitosan have shown antifungal activity, particularly against *C. albicans*, in free form of the polymer[2] or in its derivates[6,16,17]. The formulation of chitosan in solution of nanoparticles can be readily prepared by ionic gelation method using polyanions, such as tripolyphosphate (TPP). The ionic gelation method involves electrostatic interaction between the positively charged amino group of chitosan and the negatively charged polyanions[18]. Ing et al.[2] showed that of chitosan nanoparticles solution exhibited higher antifungal activity against *C. albicans* compared to the free form of chitosan, due to the higher affinity of nanoparticles to bind to fungal cells. However, this study was conducted

against planktonic cells, and it would be interesting to evaluate the effect of chitosan nanoparticles solution against *C. albicans* biofilm.

The antimicrobial activity of chitosan is observed against a variety of microorganisms, including fungi, algae and bacteria[2,3,4,5,6]. The antifungal activity occurs by the polycationic nature of chitosan that provides strong interaction with negative charges on the fungi cell wall[2,7].

In this context, there also was no study about the effect of chitosan nanoparticles solution on the physical properties of acrylic resin, used as base of denture, and on multi-species biofilm of *Candida* spp. developed on acrylic resin surface. Thus, the aim of this study was: 1) to synthesize a nanoparticle chitosan solution and to characterize this solution by Fourier Transformation Infra-Red (FTIR) analysis and Transmission Electron Microscope (TEM); 2) to evaluate its antifungal activity on planktonic fungal cells and on initial adherence, formation biofilm and reduction of *C. albicans* mature biofilm; 3) to evaluate the effect of nanoparticle chitosan solution against multi-species *Candida* spp. biofilm developed on acrylic resin surface and the surface properties (roughness and microhardness) of this material in contact with the solution during 48 h and 5 days.

2. MATERIALS AND METHODS

2.1 Preparation of chitosan nanoparticle solution

Low molecular weight (LMW, MW =70 kDa) chitosan powder (Sigma-Aldrich, São Paulo, SP, Brazil) with 75–85% degree of deacetylation was used. A concentration of 5% w/v chitosan solution was prepared by dissolving 0.05 g in 10 mL of 1% v/v acetic acid solution. The solution remained in magnetic stirring for 24h. After, the solution was filtered by paper filter for subsequent use. The nanoparticles were prepared by ionic gelation method via the interaction with TPP polyanion[18]. A concentration of 2.4 % w/v TPP solution was prepared under magnetic stirring for 30 min at room temperature. The volume of 3 mL of the TPP solution was added in 10 mL of chitosan solution with an infusion pump with dripping of 1000 mL/min. The solution was maintained under continuous stirring at 700 rpm for more 30 min. Later, the pH of the solution was adjusted to 5.5 with NaOH to confirm that acidic condition would not interfere with the antifungal activity[7]. The final concentration of nanoparticles chitosan solution (ChNP) was 3.85 mg/ mL.

2.2. Characterization of chitosan and nanoparticles formation

The chitosan used was evaluated by Fourier Transformation Infra-Red (FTIR) spectrophotometer (IRPrestige-21, Shimadzu, Kyoto, Japan). Solid samples of chitosan and TPP were diluted in KBr:sample in 100:1 mg, respectively, and pressed to form a pellet of about 1 cm of diameter. The pellets were subjected to analysis by transmittance of the infrared beam. The FTIR spectra was obtained at a frequency range of 400 - 4000 cm^{-1}, with resolution of 4 cm^{-1} and an average of 20 scans. The nanoparticles formation was evaluated by transmission electron microscopy (TEM) (Morgagni G20 – FEI, Oregon, USA). An aliquot of 50 µL of nanoparticles chitosan solution was dripped into a copper gride covered with formvar and carbon film. The grid remained in a desiccator for 24 h for drying. After the morphology and size of chitosan nanoparticles were observed using a voltage of 100kV acceleration.

2.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC and MFC of ChNP were tested against *C. albicans* ATCC 60193, *C. albicans* ATCC 10231, *C. albicans* CBS 562, *C. tropicallis* CBS 94 and *C. krusei* CBS 73. The MIC was determined by the microdilution technique using 96-well microplates (KASVI, Curitiba, PR, Brazil) according to Clinical and Laboratory Standard Institute[19]. A total of 100 µl of 2-fold concentrated Sabourand Dextrose Broth (SDB) (SDB, KASVI, Curitiba, PR, Brazil) was distributed in each well, followed by 100 µL of test solution, ChNP at initial concentrations of 3,8500 µg/mL and sodium hypochlorite 1% (NaOCl) (Sigma-Aldrich, São Paulo, Brazil). Fungal inoculum were prepared (OD_{530nm}= 0.08–0.1) and diluted in SDB to reach a final concentration of 2.5 x 10^3 CFU/mL[20]. Then 100 µL of inoculum were dispensed into each well according each strain. Tests were performed in triplicate and plates were incubated at 37°C for 24h. The medium sterility, yeast viability and the acetic acid + TPP solution were also evaluated. MIC was considered as the lowest concentration able to inhibit visible growth of the strains. In order to confirm the presence of viable or non-viable microorganisms, 10 µl of TTC dye (2,3,5 triphenyl tetrazolium chloride) was used, which reflects the activity of dehydrogenase enzymes involved in cellular respiration, staining live samples in red[21]. After 24 h of incubation, visual reading was performed.

The MFC was determined after reading of MIC by collecting aliquots of 50 µL from the subcultures corresponding to MIC, MIC x 2 and MIC x 4 and inserting them on Petri plates containing Sabouraud's Dextrose Agar (SDA) (KASVI, Curitiba, PR, Brazil). The

plates were incubated at 37ºC for 24 h. The MFC was considered the lowest concentration of the solutions that allowed no visible growth in the subculture[19]. The MFC/MIC ratio was calculated and it was determined the effect fungiostatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) of each solution[22].

2.4 Time-kill assays

C. albicans ATCC 60193 fungal cells (2 x 10^3 CFU/mL) were incubated in 96-well plate, according to microdilution technique[19], which was added ChNP and NaOCl solutions at MIC, MIC x 2 and MIC x 4 concentrations. Microplates were incubated at 37ºC for 24 h. Controls of culture medium sterility and yeast viability were also performed. After incubation, 10 µL aliquots of each concentration of solutions were seeded on SDA plates at predetermined time intervals (0, 1, 2, 4, 6, 8, 12 and 24 hours). Plates were incubated at 37ºC for 24 h. The number of viable cells (CFU) was counted and the values were expressed in log CFU/mL. The results were represented graphically the fungal cell death curve as a function of time. The test was performed in triplicate.

2.5 Effect of ChNP on fungal micromorphology

The microculture technique was employed using agar-cornmeal (CA) (KASVI, Curitiba, PR, Brazil) in a moist chamber/Petri dish method[23]. *C. albicans* cells (ATCC 60193) (2 x 10^3 CFU/mL) were used in this test. ChNP and NaOCl were used at MIC, MIC x 2 and MIC x 4 concentrations.

The solutions (ChNP and NaOCl) in different concentrations were added to the agar culture medium in liquid phase. Then, 1 mL of agar with the solutions were placed in a glass slide containing a sterile glass supporter. After solidification, two parallel striations were covered with sterile coverslips containing the tested yeast, and a glass coverslip was placed over the medium. To avoid desiccation, the glass slide with medium and yeast were maintained in a moist chamber with 2 mL of distilled water on a piece of sterile filter paper. The system was incubated at 37ºC for 48 h. The evaluation of fungal micromorphology was performed using light microscopy at 40X magnification and it was observed the presence of typical structures such as pseudohyphae, blastoconidia and chlamydoconidia.

2.6 Evaluation of antifungal activity of ChNP on C. albicans adherence, biofilm formation and reduction of mature biofilm

The anti-biofilm activity of ChNP at CIM, CIM x 2 and CIM x 4 concentrations against *C. albicans* ATCC 60193 were evaluated at three different experiments: on the initial adherence , biofilm formation and reduction of mature biofilm.

The time of contact of ChNP and NaOCl solutions with the fungal cells were used to simulate the two forms of clinical application: 1) a short contact period with the solutions for 1 min, every 8 h, simulating the immersion of the denture into the solution during the time of daily oral hygiene; 2) Prolonged contact time with solutions for 8 consecutive hours, simulating the soaking of denture into the solution overnight. Seven groups were formed according to the time of addition of solutions in the culture medium; the contact time of solutions with fungal cells and elapsed time for biofilm formation; and the time dispensed for reading in the absorbance reader (Figure 1).

The tests were performed in triplicate. NaOCl was used as positive control at concentrations of the MIC, MIC x 2 and MIC x 4 in all groups. Viability and growth controls were performed following the same methodology for each group.

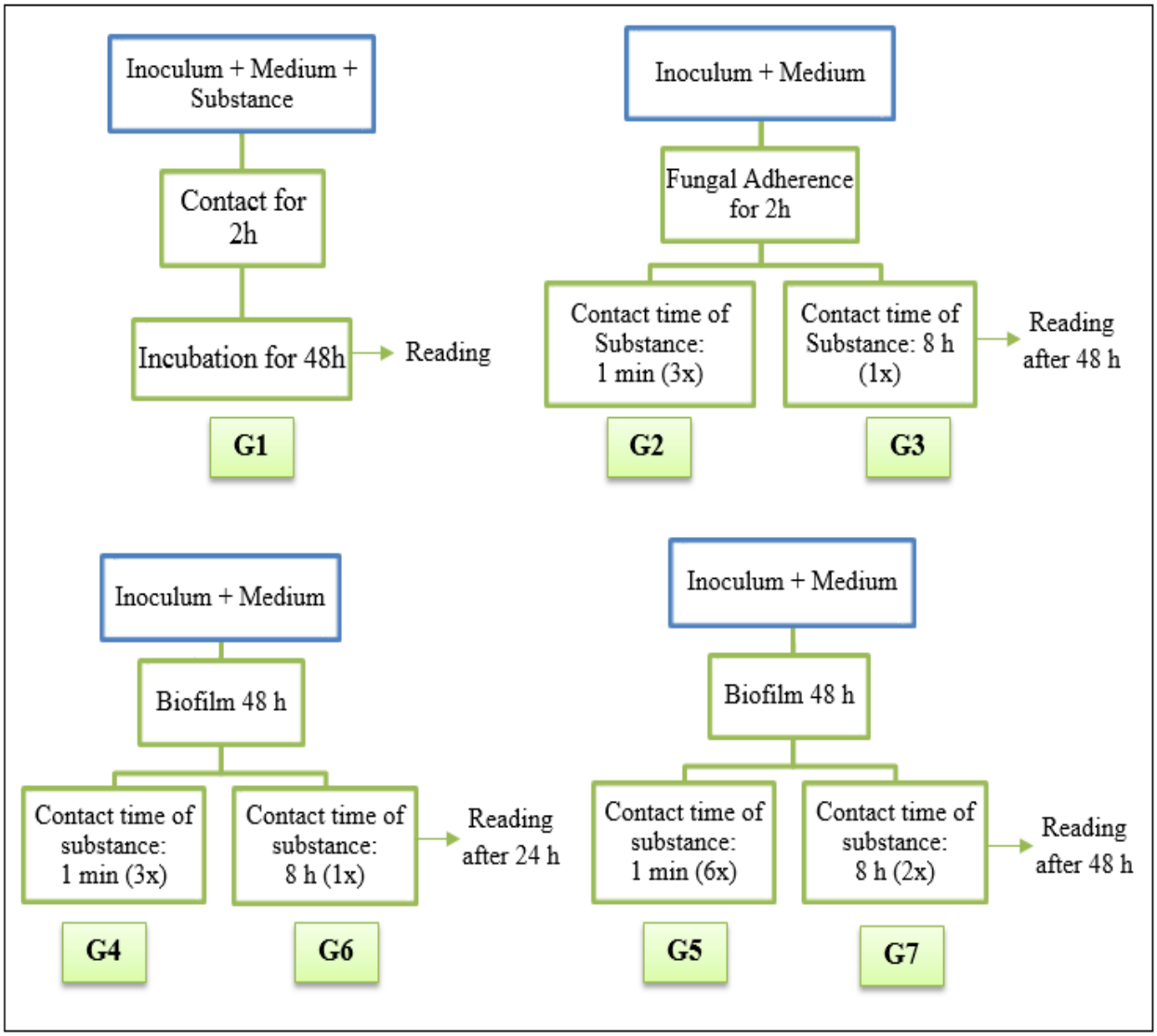

Figure 1: Characterization of groups formed to evaluate the anti-biofilm activity of the solutions according to the time of addition of solutions in the culture medium; the contact time of solutions with fungal cells and elapsed time for biofilm formation; and the time dispensed for reading in the absorbance reader. G1: effect of ChNP on initial adherence of *C. albicans*; G2 and G3: effect of ChNP on *C. albicans* biofilm formation; G4, G5, G6 and G7: effect of ChNP on reduction of *C. albicans* mature biofilm

2.6.1. *Evaluation of ChNP on initial adherence of C. albicans*

In a 96-well plates, 100 µL of SDB containing a suspension of 2.5 x 10^6 CFU/mL was transferred to each well. Then, 100 µL of solutions (ChNP and NaOCl) in CIM, CIM x 2 and CIM x 4 concentrations were added to the wells. The plates were incubated for 2h at 37°C for fungal adherence. The effect of solutions in contact with yeast cells during 2h of initial fungal adherence was evaluated (Group 1 - G1).

After 2h of adhesion with the solutions, the suspension of fungal cells were removed from each well. The wells were washed twice with 200 µL of phosphate buffered saline (PBS) to remove non-adherent cells and 100 µL of SDB medium was transferred into each well. The plate was incubated at 37 °C for 48 h.

After incubation, the content of wells were discarded and they were washed twice and dried for 45 min at room temperature. Then 200 µL of 0.4% crystal violet (Sigma-Aldrich, São Paulo, SP, Brazil) was added to each well and incubated for 45 min. The wells were washed and immediately decolorized with 200 µL 95% ethanol. After 45 min, 100 µL of the decolorized solution was transferred to a new plate for reading in absorbance reader at 600 nm (GloMax-Multi, PROMEGA, Madison, Wisconsin, USA)[20].

The absorbance values obtained were calculated as percentage of inhibition (% inhibition). The % inhibition was measured indirectly relative to the values obtained of the yeast growth group, which was assigned a value of 100% fungal adherence[20].

2.6.2 Evaluation of ChNP on the biofilm formation of C. albicans

In 96-well plates, 100 µL of *C. albicans* suspension (2.5 x 10^6) were transferred to each well and incubated for 2 h at 37 °C. After initial adherence, the fungal suspension was aspirated from each well and the plate washed twice with 200 µL of PBS to remove the non-adhered cells. After 100 µL of SDB medium was transferred to each well. Then, 100 µL of each solution (ChNP and NaOCl at MIC, MIC x 2 and MIC x 4 concentrations), was added to wells.

For Group 2 (G2) the solutions remained in contact with fungal cells for 1 min. This contact time was repeated 3x every 8 h. After each contact, the wells were washed twice with 200 µLPBS and 100 µL SDB were added to each well. The plate was incubated for 48 h at 37 °C for later reading.

For Group 3 (G3) the solutions remained in contact with fungal cells for 8 consecutive hours The wells were washed twice with 200 µL PBS and 100 µL SDB was added to each well. The plate was incubated for 48 h at 37 °C for later reading.

After the incubation, the violet crystal staining was performed and the plates were conducted for absorbance reader, as previously described. The % inhibition of biofilm formation indirectly relative the values obtained of the yeast growth group, which was assigned a value of 100% fungal biofilm formation[20].

2.6.3 Evaluation of ChNP on the reduction of C. albicans mature biofilm

The *C. albicans* adherence was performed as previously described 2.6.2. The plates were incubated for 48 h at 37 °C for mature biofilm formation. After, the medium was aspirated and the wells washed twice with 200 µL PBS. An aliquot 100 µL of SDB and 100 µL of each solution (ChNP and NaOCl at MIC, MIC x 2 and MIC x 4 concentrations) were inserted in each well.

For Groups 4 and 5 (G4 and G5), the solutions remained in contact with biofilm for 1 min. The wells were washed twice with 200 µL of PBS and 100 µL of SDB was added to each well. The plate was incubated at 37 °C. For G4, the solutions were inserted in wells for 3x, every 8h, totalizing 24h incubation for later reading. For G5, the solutions were inserted in wells for 6x, every 8h, with 3 applications for day, totalizing 48h incubation for later reading.

For Groups 6 and 7 (G6 and G7), the solutions remained in contact with biofilm for 8 consecutive hours. After this period, the wells were washed twice with 200 µL of PBS and 100 µL of SDB were added to each well. The plate was incubated at 37 °C. For G6 the reading was performed after 24 h of solution addition, totalizing 8h of solution contact time with *C. albicans* biofilm. For G7 the solutions were inserted in wells one time a day for 8 consecutive hours, during 48h, totalizing 16h of solution's contact with *C. albicans* biofilm. After 48h incubation, the reading was performed.

The violet crystal staining of biofilm was performed and the plates were conducted for absorbance reader, as previously described. The % inhibition of mature reduction biofilm was calculated indirectly relative to the values obtained of the yeast growth group, which was assigned a value of 100% fungal biofilm formation[20].

2.7 Effect of ChNP on Candida spp. multi-species biofilm colonized on acrylic resin and in its surface properties

2.7.1 Preparation of acrylic resin specimens

Forty-eight cylindrical of heat-cured acrylic resin (Vipi Wave; VIPI, Pirassununga, SP, Brazil) were obtained by wax patterns with the same dimensions (4 x 2 mm). The wax pattern was invested in a fiberglass flask with a glass plate (5mm) and type III dental stone (Herodent, Vigodent S/A Ind. Com, Rio de Janeiro, Brazil). After the investing material had set, the flask halves were separated and wax was removed with boiling water. A coat of acrylic separating film (Cel-Lac; S.S. White, Rio de Janeiro, RJ, Brazil) was applied on the

stone surface. The heat-processed acrylic resin was mixed according to the manufacturer's instructions, and packed into the stone mold. A hydraulic press (PM 2000, Nova Techno, Várzea Paulista, SP, Brazil) was used for packing the denture base resin at 1250 kgf, maintained for 30 min.

Specimens were then polymerized on a microwave with 900 W with 20 min with at 20% of the power and 5 min with a 60% of the power, according to the manufacturer's instructions. After, the specimens were removed of flasks and subjected to finishing with tungsten grinder (1508 Edenta AG, Haupistrasse, Switzerland) and polishing with 220, 330, 600 and 1200-grit wet/dry sandpapers and felt discs embedded in pumice slurry. The initial surface roughness (Ra) and Vickers microhardness (VHN) measurements were perfomed and the specimens were sterilized with ethylene oxide.

2.7.2 Surface roughness measurements

The specimens were fitted to the surface roughness-measuring instrument (TR200, Digimess, São Paulo, SP, Brazil). In each specimen, the same examiner obtained three successive measurements in the central area in different directions. The mean surface roughness values (Ra) were obtained and expressed in micrometers. The roughness testing was conducted before and after biofilm formation (48h and 5 days).

2*.7.3. Surface microhardness measurements*

The microhardness measurements were performed with a microhardness tester (HMV II; Shimadzu Corporation, Kyoto, Japan) using a Vickers indenter (VHN) and a load of 50 g with a dwell time of 10 s. Five indentations were made in each specimen, at least 50 µm apart, and the mean VHN value was obtained. The test was conducted before and after biofilm formation (48h and 5 days).

2.7.4 Candida spp. multi-species biofilm formation and ChNP application

The *Candida* strains used to develop the multi-species biofilm were *C. albicans* ATCC 60193, *C. tropicallis* CBS 94 and *C. krusei* CBS 73. The microorganisms were cultivated in SDB medium at 37 °C for 24 h and the number of cells was adjusted to approximately 1 x 10^7 CFU/mL. Next, the final suspension species was prepared by mixing equal volume 5 mL of each inoculum in a glass tube.

Specimens were divided into four groups (n=12): GI– Growth control (no antifungal solution) ; GII– ChNP at CIM concentration; GIII – ChNP at CIM x 4 concentration and

GIV – 1% NaOCl. In a 24-well plate, the specimens of acrylic resin were randomly added to each well and 150 µL of *Candida* spp. multi-species suspension were carefully placed on its surface and the plates were incubated for 2 h at 37 °C for initial adherence. After the adhesion stage, the fungal suspension was aspirated from each specimen and washed twice with 2 mL of PBS. In each well, 2 mL of SDB was transferred and the plates were incubated for 24 h at 37 °C for biofilm formation.

After 24 h, the culture medium was aspirated and the wells were washed carefully twice with 2 mL of PBS to remove non-adherent cell. Following, 1 mL of SDB and 1 mL of the solution were added to wells, according to each group. The solutions remained in contact with the biofilm for 8 h consecutive hours to simulate the time of immersion of denture in disinfectant solution. After this period, the wells were washed twice with 2 mL of PBS, and finally, 2 mL of SDB were added to each well and the plate was incubated at 37 °C. This procedure was performed every day during 5 days. The CFU of each group was performed after 48h (n=6 of each group) and after 5 days (n=6 of each group).

After the incubation time, the specimens were placed in tubes containing 2 mL of PBS and stirred for 60 s. Then, they were serially diluted (10^{-1} to 10^{-6}) and an aliquot of 10 µL were seeded on SDA plates, which were incubated at 37°C for 24 h. Thereafter, the counting of viable cells (CFU) was carried out and the values were expressed as CFU/mL.

The final measurements of roughness surface and microhardness after biofilm formation were conducted in each specimen, as previously described.

2.7.5 Statistical analysis

Data analysis was performed with the GraphPad Instat computer program, version 2.0 (GraphPad software, CA, USA), at a level of significance of $\alpha=0.05$. The % inhibition of adherence, biofilm formation and reduction were analyzed using Kruskal-Wallis test followed by Dunn post-test ($p < 0.05$) to compare % inhibition of the same solution among the different concentrations (CIM, CIM x 2 and CIM x 4). For comparison between the solutions at the same concentration Mann Whitney test was applied. For the tests conducted on acrylic resin, ANOVA one-way and Tukey tests were performed for statistical comparisons of CFU/mL among solutions after 48 h and 5 days of biofilm formation and for comparisons of Ra and VHN measurements among solutions in each experimental period (before or after biofilm). The paired t-test was used to compare Ra and VHN measurements before and after biofilm formation.

3. RESULTS

3.1 FTIR analysis

The FTIR spectrum of powder TPP (A), powder chitosan (B), and chitosan nanoparticle (lyophilized) (C) are illustrated in Figure 2. It was observed in chitosan nanoparticle infrared spectrum a stretching of C = O in 1631 cm^{-1}, N-H in 1548 cm^{-1} and C-N in 1408 cm^{-1} that representing the secondary amide. Absorptions of primary amine were observed between 1138 cm^{-1} and 952 cm^{-1} due to stretching in C-N and deformation out of the plane in N-H 896 cm-1 (Figure 1C). Therefore, there was an interaction between the two solutions (chitosan and TPP), since there was an overlap of infrared spectra.

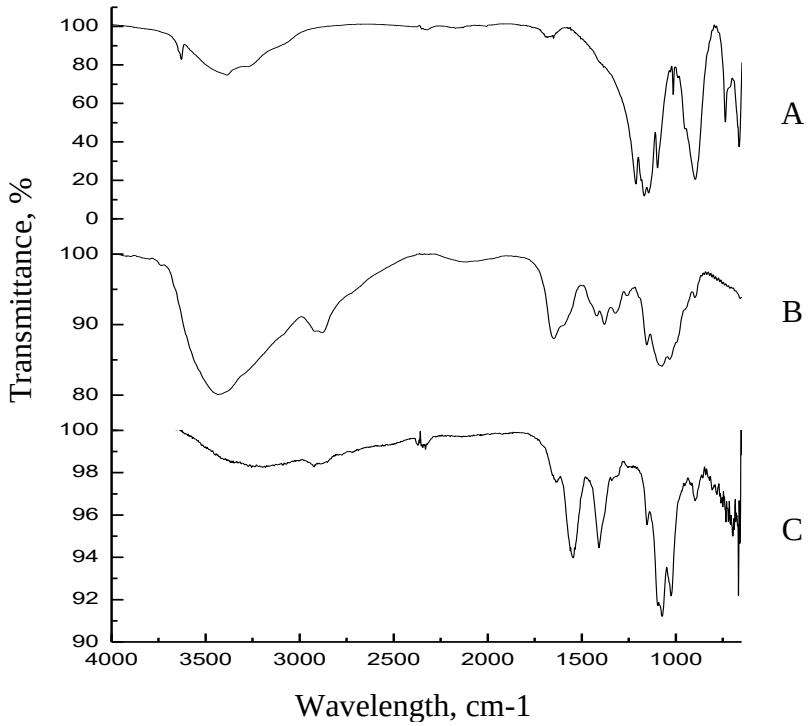

Figure 2: FTIR spectra of powder TPP (A), powder chitosan (B), and chitosan nanoparticle (lyophilized) (C)

3.2 TEM analysis

There was formation of chitosan nanoparticles created by ion gelation with TPP (Figure 3). The particles were rounded and had regular size. Some particles tended to clump into larger particles. The average size of particles was about 0.05 µm (50 nm).

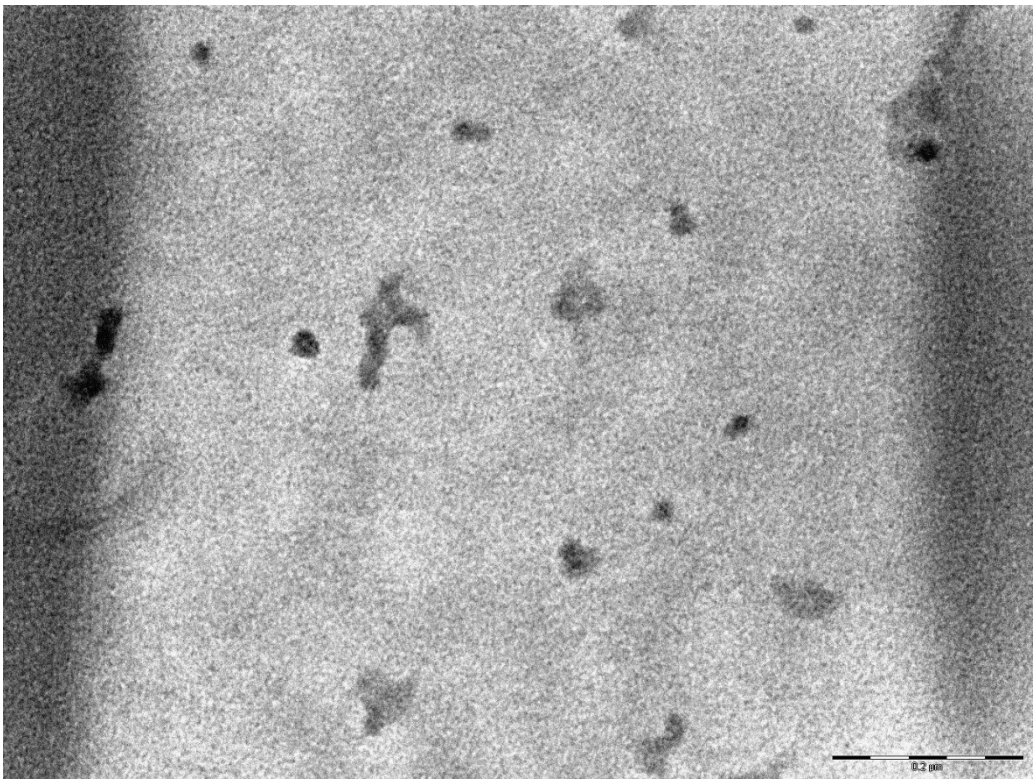

Figure 3. TEM image of chitosan nanoparticles formation created by ion gelation with TPP. —: Reference bar: 0.2 µm.

3.3 Determination of MIC and MFC

MIC and MFC values of ChNP and NaOCl on *C. albicans*, *C. tropicallis* and *C. krusei* are illustrated in Table 1. For ChNP, MIC values found ranged between 15.04 µg/mL for *C. albicans* ATCC 60193 and *C. albicans* ATCC 10231; 60.15 µg/mL for *C. albicans* CBS 562. For *C. tropicallis* and *C. krusei* MIC value was 30.08 µg/mL to both yeasts. The concentration of 30.08 µg/mL can be defined as the concentration able to inhibit 83.3% of the tested strains ($MIC_{83.3\%}$). For the MFC, the concentrations values ranged between 15.04 to 120.31µg/mL.

The ratio MFC/MIC found for the all concentrations of solutions demonstrated a fungicidal effect against *Candida* spp. tested.

Table 1: Antifungical activity of chitosan nanoparticle solution (ChNP) and sodium hypochlorite (NaOCl) on *Candida* spp.

Strain	Compound	MIC (µg/mL)	MFC (µg/mL)	MFC/MIC	Antifungical activity
Candida albicans ATCC 60193	ChNP	15.04	15.04	1	Fungicidal
	NaOCl	625	625	1	Fungicidal
Candida albicans ATCC 10231	ChNP	15.04	15.04	1	Fungicidal
	NaOCl	625	625	1	Fungicidal
Candida albicans CBS 562	ChNP	60.15	120.31	2	Fungicidal
	NaOCl	312.5	625	2	Fungicidal
Candida tropicallis CBS 94	ChNP	30.08	30.08	1	Fungicidal
	NaOCl	1250	1250	1	Fungicidal
Candida krusei CBS 73	ChNP	30.08	30.08	1	Fungicidal
	NaOCl	312.5	312.5	1	Fungicidal

3.4 Time-kill assays

The effect of ChNP and NaOCl in inhibiting *C. albicans* cell growth was verified. It was observed that at all concentrations of the solutions (MIC, MIC x 2 and MIC x 4) were able to reduce the number of CFU/mL when compared to the control group. The concentrations of MIC and MIC x 2 of ChNP at 0 and 1h was effective in inhibit *C. albicans* with reduction similar to NaOCl (Figs. 4 and 5). However, after 4 h until 12 h ChNP had lower inhibition of *C. albicans* growth compared to NaOCl, but showed higher inhibition of growth than growth control (Figs. 4 and 5). After 24 h, none of solutions at the MIC and MIC x 2 concentrations were able to inhibit fungal cell growth. At MIC x 4, ChNP was similar to NaOCl, showing higher inhibition of *C. albicans* growth compared to other concentrations (Fig. 6) A significant decreasing of yeast growth was visualized after 2 h until 6 h, and none fungal cell growth was verified after 4h (Fig.6).

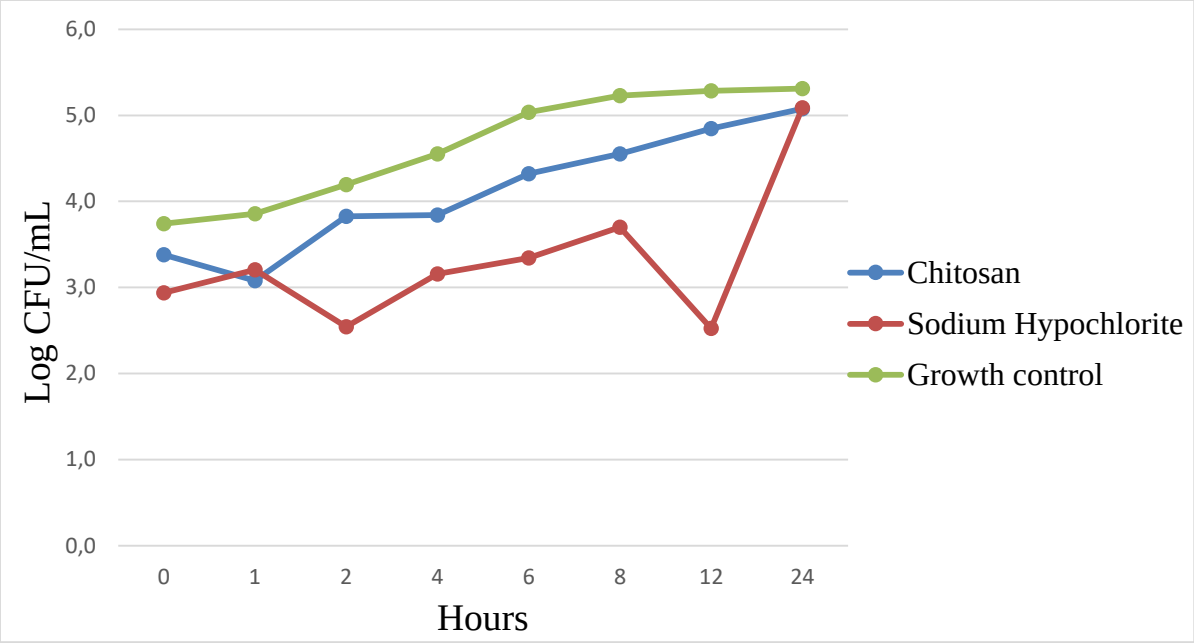

Figure 4: Time Kill *Candida albicans* ATCC 60193 at MIC concentrations of ChNP (30.08 µg/mL) and NaOCl (625 µg/mL)

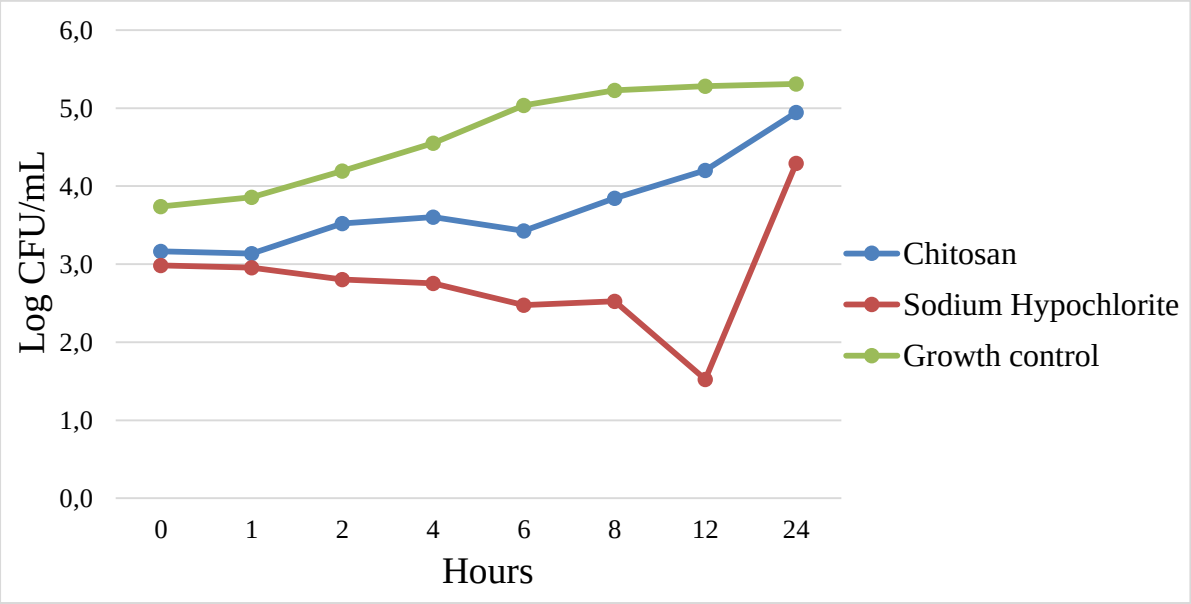

Figure 5: Time Kill *Candida albicans* ATCC 60193 at MIC x 2 concentrations of ChNP (60.15 µg/mL) and NaOCl (1,250 µg/mL)

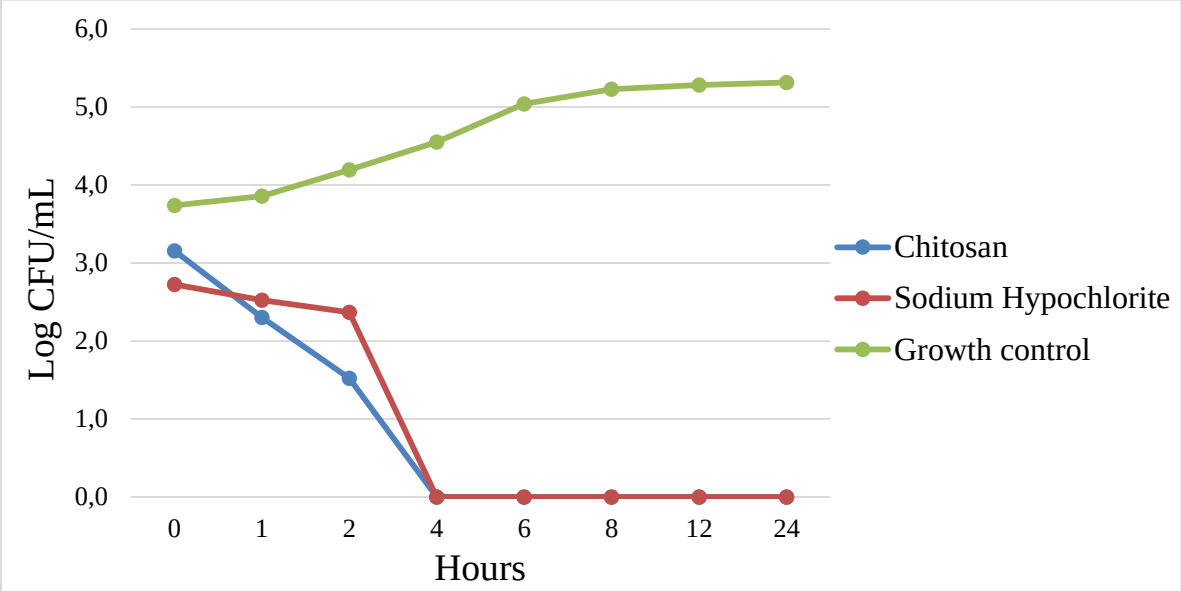

Figure 6: Time Kill *Candida albicans* ATCC 60193 at MIC x 4 concentrations of ChNP (120.31µg/mL) and NaOCl (2,500 µg/mL)

3.5 Effect of ChNP on fungal micromorphology

Microscopical evaluation of *C. albicans* ATCC 60193 microculture identified the presence of inherent characteristics of the fungal cells with pseudohyphae, blastoconidia and chlamydospores in abundance, which reflects viability and normal capacity of morphogenesis (Fig. 7A). ChNP was able to reduce the formation of filamentous yeast form that was directly proportional to the increase of concentration (Fig. 7B). For NaOCl, MIC x 2 (1,250 µg/mL) and MIC x 4 (2,500 µg/mL) concentrations showed total inhibition of pseudohyphae and chlamydoconidia, only blastoconidia was observed (Fig. 7C).

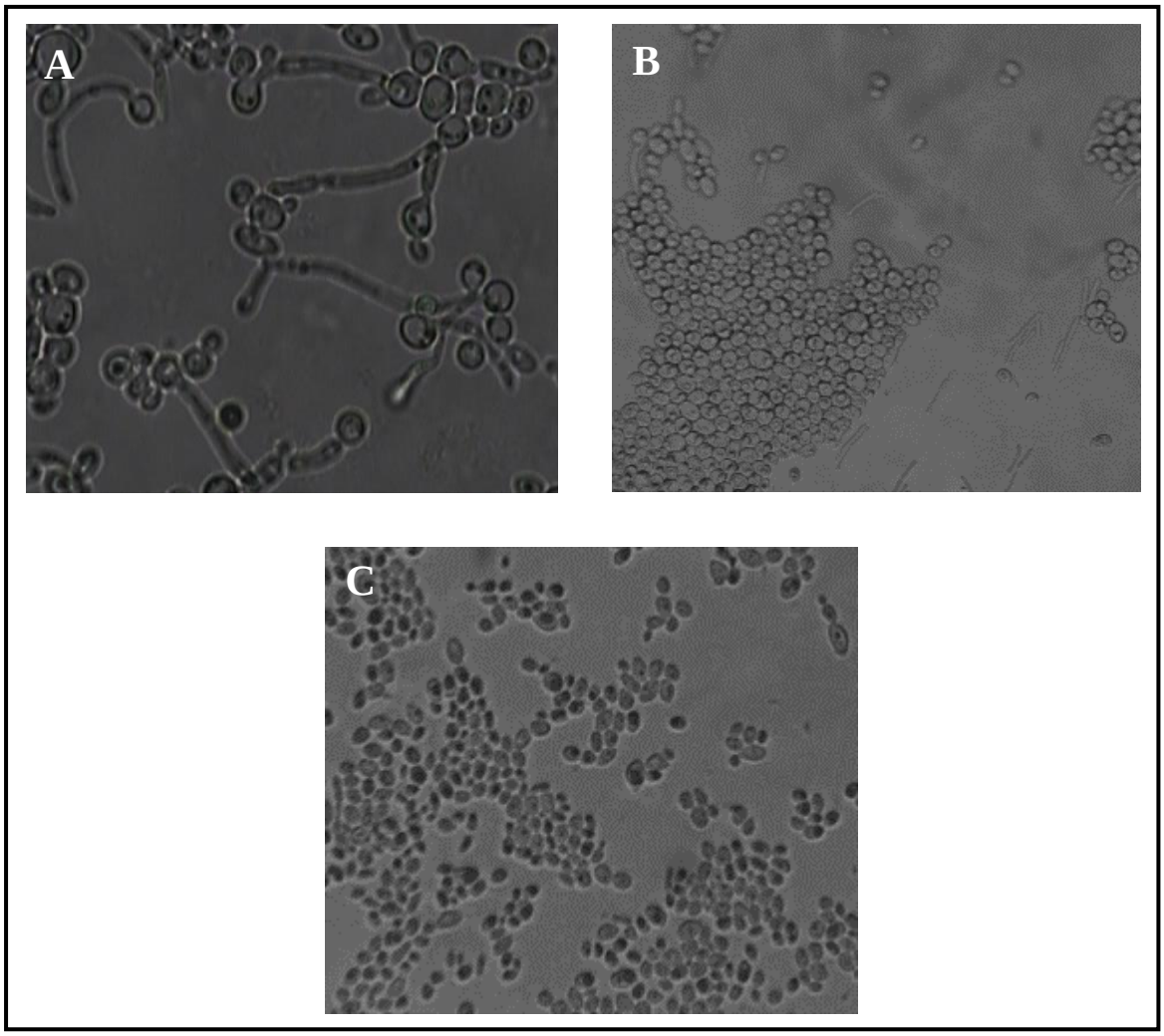

Figure 7. Micromorphology of *C. albicans* (ATCC 60193). A: growth control; B: chitosan nanoparticle solution (CIM x 2 = 60.15 µg/mL); C: sodium hypochlorite (CIM x 2 = 1,250 µg/mL)

3.6 Effect of ChNP on adherence, formation and reduction of *C. albicans* biofilm

The % inhibition (% I) of ChNP on initial adherence of *C. albicans* (G1) increased with the increase of its concentration. NaOCl showed higher % I on initial adherence than ChNP at MIC and MIC x 2 ($p=0.05$). However, there was no statistical difference between ChNP and NaOCl at MIC x 4 ($p=0.05$) (Table 2). The % I on biofilm formation with 1 min contact time of the solutions (G2), ChNP demonstrated similar % I in all concentrations and higher % I at MIC x 4 compared to NaOCl ($p=0.002$) (Table 2). The % I on biofilm formation after contact time of 8 h (G3) showed that ChNP was more effective in inhibit *C. albicans* biofilm formation than NaOCl in all concentrations ($p<0.05$) (Table 2).

After 48h mature biofilm formation ChNP and NaOCl were applied in different times and incubated for 24h, totalizing 72h mature biofilm. Thus, ChNP showed higher %I of *C. albicans* mature biofilm than NaOCl at MIC x 2 and MIC x 4 in G4 (contact time: 3 x 1min)

(p= 0.004) and at all concentrations in G6 (contact time:1 x 8h) ($p<0.05$) (Table 3). However, after solution's application and 48h incubation, totalizing 96 h mature biofilm, G5 (Contact time: 6x 1min) and G7 (Contact time: 2x 8h) showed that ChNP at MIC and MIC x 2 concentrations did not have inhibition against *C. albicans* mature biofilm (Table 3). For G5 group, ChNP at MIC x 4 showed higher % I than NaOCl, but for G7 group NaOCl demonstrated better % I (Table 3). It also was demonstrated that the % I of ChNP on mature biofilm was proportional to its concentration (Table 3).

Table 2. Inhibitory effects of Chitosan nanoparticles and Sodium hypochlorite solutions on the initial adherence and the formation of *C. albicans* (ATCC 60193) biofilm after different contact times and 48 h of incubation. Values were expressed as median (minimum / maximum values) of percentage of inhibition (% I).

Concentration	Median % I on initial adherence		Median % I on formation biofilm			
	Group 1 (Contact time: 2h)		Group 2 (Contact time: 1 min)		Group 3 (Contact time: 8h)	
	Chitosan Nanoparticles	Sodium hypochlorite	Chitosan Nanoparticles	Sodium hypochlorite	Chitosan Nanoparticles	Sodium hypochlorite
CIM	25.9 $^{B*, b**}$ (25.1/32.4)	53.6 $^{A, a}$ (46.7/55.0)	46.0 ^{A,a} (44.5/47.5)	47.9 ^{AB,a} (45.7/50.2)	50.2 ^{B,a} (49.8/50.6)	45.7 ^{B,b} (41.8/49.7)
CIM x 2	36.5 $^{AB, b}$ (34.5/37.0)	57.3 $^{A, a}$ (51.7/62.9)	41.7 ^{A,b} (40.4/47.4)	51.6 ^{A,a} (50.8/52.4)	56.6 ^{A,a} (50.5/57.1)	47.7 ^{AB,b} (47.1/48.3)
CIM x 4	43.7 $^{A, a}$ (41.1/47.8)	50.6 $^{A, a}$ (47.2/54.0)	51.1 ^{A,a} (40.7/51.4)	39.6 ^{B,b} (39.1/40.4)	57.8 ^{A,a} (55.4/60.3)	53.1 ^{A,b} (51.8/54.4)

* Different uppercase letters in the same column indicate statistically significant difference ($p<0.05$) among the concentrations of the same solution (Chitosan nanoparticles or Sodium hypochlorite) by Kruskal-Wallis test followed by Dunn post-test ($\alpha = 0.05$).
** Different lowercase letters in the same line indicate statistically significant difference ($p<0.05$) between the chitosan nanoparticles and sodium hypochlorite at the same concentration against *C.albicans* by Mann Whitney test ($\alpha = 0.05$).
Note: Tests were performed in triplicate of three independent experiments.

Table 3. Inhibitory effects of Chitosan nanoparticles and Sodium hypochlorite solutions on the reduction of *C. albicans* (ATCC 60193) mature biofilm (48h) after different contact times and periods of incubation (24 or 48h). Values were expressed as median (minimum / maximum values) of percentage of inhibition (% I).

Concentration	**Median % I on reduction of mature biofilm**							
	Group 4 (Contact time: 3x 1min/24h)		**Group 5 (Contact time: 6x 1min/48h)**		**Group 6 (Contact time:8h/24h)**		**Group 7 (Contact time:2x 8h/48h)**	
	Chitosan Nanoparticles	**Sodium hypochlorite**	**Chitosan Nanoparticles**	**Sodium hypochlorite**	**Chitosan Nanoparticles**	**Sodium hypochlorite**	**Chitosan Nanoparticles**	**Sodium hypochlorite**
CIM	60.3 ^{AB*} (59.5/61.2)	-	-	39.9 ^{A} (32.9/41.5)	62.3 $^{AB, a}$ (61.3/63.3)	54.0 $^{A, b}$ (50.4/57.4)	-	46.3 ^{A} (46.3/56.1)
CIM x 2	58.5 $^{B, a**}$ (57.8/59.3)	12.5 $^{B, b}$ (11.4/13.7)	-	32.5 ^{A} (29.6/36.2)	52.2 $^{B, a}$ (52.2/52.7)	46.8 $^{A, b}$ (43.2/50.5)	-	47.9 ^{A} (46.3/55.9)
CIMx4	63.0 $^{A, a}$ (61.3/64.7)	42.7 $^{A, b}$ (33.8/51.7)	54.4 ^{a} (49.7/60.5)	32.4 $^{A, b}$ (29.5/32.5)	66.1 $^{A, a}$ (65.6/66.7)	46.3 $^{A, b}$ (45.2/53.9)	36.6 ^{b} (31.0/36.8)	49.8 $^{A, a}$ (47.6/52.0)

* Different uppercase letters in the same column indicate statistically significant difference ($p<0.05$) among the concentrations of the same solution (Chitosan nanoparticles or Sodium hypochlorite) by Kruskal-Wallis test followed by Dunn post-test ($\alpha = 0.05$).

** Different lowercase letters in the same line indicate statistically significant difference ($p<0.05$) between the chitosan nanoparticles and sodium hypochlorite at the same concentration against *C.albicans* by Mann Whitney test ($\alpha = 0.05$).

Note: Tests were performed in triplicate of three independent experiments, ‘‘-’’ means ‘‘no inhibition of adherence’’

3.7. Effect of ChNP on *Candida* multi-species biofilm on acrylic resin and on its surface properties

The mean number of viable multi species *Candida* cells (CFU/mL) adhered to the acrylic resin surface after biofilm formation for 48 h and 5 days are illustrated in Figure 8 and 9, respectively. After 48 h, ChNP at MIC x 4 and 1% NaOCl showed significant reduction of fungal cells compared to control group ($p<0.001$). ChNP at MIC concentration did not shown reduction of CFU/ mL compared to control group (Fig. 8). After 5 days (Fig. 9), all solutions were able to decrease CFU/mL, NaOCl showed the highest reduction of *Candida* spp. on biofilm, followed by ChNP at MIC x 4, ChNP at MIC and control group ($p=0.001$).

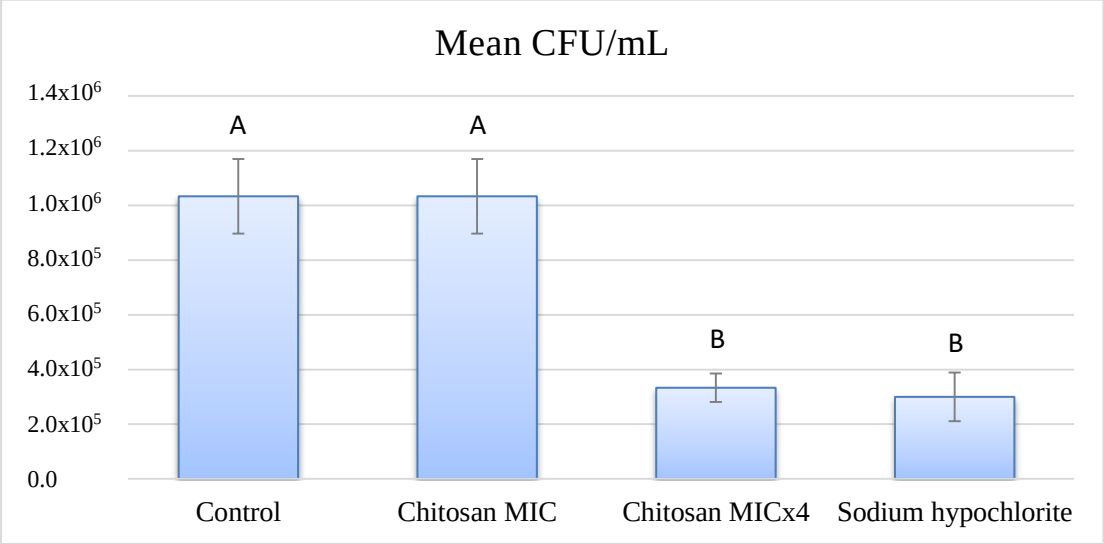

Figure 8. The effect of ChNP at MIC and MIC x 4 and 1% NaOCl on *Candida* multi-specie biofilm composed by *C. albicans*, *C. tropicallis* and *C. krusei* on acrylic resin for 48 h. Error bars represent standard deviations ($\alpha=0.05$). Different capital letters denote significant differences among groups (Anova One- way and Tukey tests, $p=0.05$)

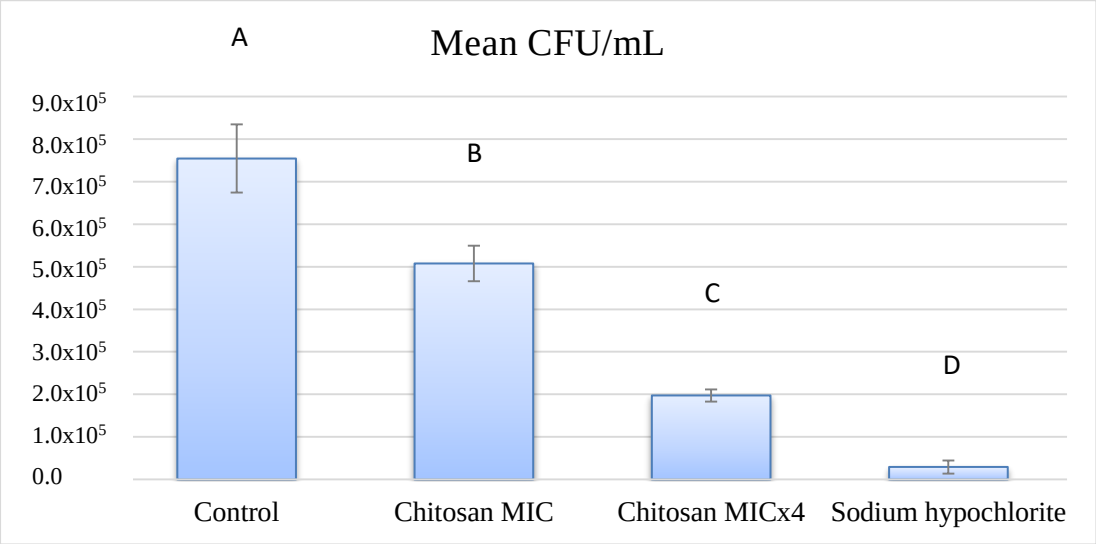

Figure 9. The effect of ChNP at MIC and MIC x 4 and 1% NaOCl on *Candida* multi-specie biofilm composed by *C. albicans*, *C. tropicallis* and *C. krusei* on acrylic resin for 5 days. Error bars represent standard deviations (α=0.05). Different capital letters denote significant differences among groups (Anova One- way and Tukey tests, p=0.05).

The Ra values of acrylic resin specimens before and after solutions application's during biofilm formation are summarized in Table 4. There was no significant difference among solutions for Ra values before biofilm formation during 48 h and 5 days (p>0.05). After 48h biofilm, the control group showed the highest Ra value compared to other groups (p=0.03), but there was no significant difference among ChNP (MIC and MIC x 4) and 1% NaOCl groups. After 5 days biofilm, NaOCl group showed the highest Ra value without statistical difference with control group (p=0.07), and ChNP at MIC x 4 showed the lowest Ra value (p=0.02). For comparisons of Ra values before and after biofilm for each group, it was found that only the control group showed increase of Ra value after 48 h biofilm (p=0.001). After 5 days biofilm, only control and NaOCl groups showed increase of Ra value (p= 0.001).

The VHN values of acrylic resin specimens before and after solutions application's during biofilm formation are summarized in Table 5. There was no significant difference among solutions for VHN values before biofilm formation during 48 h and 5 days (p>0.05). After 48h, NaOCl group showed significant decreasing of VHN value compared to ChNP at MIC (p=0.016). After 5 days biofilm, NaOCl group showed the lowest VHN value without statistical difference with control group (p=0.07), and ChNP at MIC and MIC x 4 showed

the highest VHN value (p=0.002). However, for comparisons of VHN before and after biofilm for each group, all groups showed reduction of VHN values.

Table 4. Values of surface roughness (Ra) (mean ± standard deviation) of acrylic resin exposed to chitosan nanoparticle solution (ChNP - at MIC and MIC x 4) and sodium hypochlorite (NaOCl 1%) during *Candida* spp. biofilm formation for 48 h and 5 days.

	Biofilm 48 hours		**Biofim 5 days**	
Groups	**Before**	**After**	**Before**	**After**
Control	0.045 ± 0.008 $^{A*, b**}$	0.054 ± 0.007 $^{B, a}$	0.041 ± 0.006 $^{A, b}$	0.048 ± 0.011 $^{AB, a}$
ChNP (MIC)	0.042 ± 0.008 $^{A, a}$	0.045 ± 0.007 $^{AB, a}$	0.041 ± 0.006 $^{A, a}$	0.048 ± 0.004 $^{AB a}$
ChNP (MICx4)	0.042 ± 0.007 $^{A, a}$	0.042 ± 0.006 $^{AB, a}$	0.039 ± 0.003 $^{A, a}$	0.044 ± 0.006 $^{B, a}$
NaOCl	0.039 ± 0.009 $^{A, a}$	0.040 ± 0.010 $^{A, a}$	0.049 ± 0.009 $^{A, b}$	0.060 ± 0.008 $^{A, a}$

* Different uppercase letters in the same column indicate differences statistically significant among solutions at the same experimental period by Anova One-way test followed by Tukey post-test ($p<0.05$).
** Different lowercase letters in the same line indicate difference statistically significant in each solution before and after application of solutions during biofilm formation by pared Student T test ($p<0.05$) .

Table 5. Values of Vickers microhardness (VHN) (mean ± standard deviation) of acrylic resin exposed to chitosan nanoparticle solution (ChNP - at MIC and MIC x 4) and sodium hypochlorite (NaOCl 1%) during *Candida* spp. biofilm formation for 48 h and 5 days.

	Biofilm 48 hours		**Bofilm 5 days**	
	Before	**After**	**Before**	**After**
Control	20.7 ± 0.74 $^{A*, a**}$	20.0 ± 0.45 $^{AB, b}$	20.8 ± 0.84 $^{A, a}$	19.3 ± 0.27 $^{B, b}$
ChNP (MIC)	20.8 ± 0.34 $^{A, a}$	20.3 ± 0.28 $^{A, b}$	20.8 ± 0.66 $^{A, a}$	20.3 ± 0.62 $^{A, b}$
ChNP (MICx4)	20.6 ± 0.50 $^{A, a}$	20.1 ± 0.34 $^{AB, b}$	20.5 ± 0.32 $^{A, a}$	20.1 ± 0.14 $^{A, b}$
NaOCl	20.6 ± 0.50 $^{A, a}$	19.6 ± 0.29 $^{B, b}$	20.7 ± 0.38 $^{A, a}$	19.5 ± 0.12 $^{B, b}$

* Different uppercase letters in the same column indicate differences statistically significant among solutions the groups at the same experimental period by Anova One-way test followed by Tukey post-test ($p<0.05$).
** Different lowercase letters in the same line indicate difference statistically significant in each solution before and after application of solutions during biofilm formation by pared Student T test ($p<0.05$).

4. DISCUSSION

Chitosan has been broadly studied and the fungicidal activity to *Candida* planktonic cells is already know[1,2,3,4,6,7,24]. Ing et al. [2] showed that the application of the chitosan in the form of nanoparticles was more effective as antifungal agent against *Candida* planktonic cells than free form of chitosan. However, the present study showed an innovative information about the effect of ChNP on *Candida* biofilm and on acrylic resin surface.

It is known that chitosan nanoparticles can be prepared using many methods such as ionic gelation, complex coacervation, emulsion cross-linking and spray drying. In the present study, MET analysis (Fig. 3) showed the formation of rounded and regular chitosan nanoparticles with approximately 50 nm. The ionic gelation method was efficient to synthetize the nanoparticles, being an easy and fast technique[25]. The FTIR spectra of chitosan, TPP and nanoparticles chitosan were shown in Fig. 2. A characteristic band at 3449 cm^{-1} is attributed to –NH2 and –OH groups stretching vibration and the band for amide I at 1655 cm^{-1} is seen in the infrared spectrum of chitosan[26]. Whereas in the FTIR spectra of cross-linked chitosan the peak of 1655 cm^{-1} disappears and 2 new peaks at 1645 cm^{-1} and 1554 cm^{-1} appears. The disappearance of the band could be attributed to the linkage between the phosphoric and ammonium ions, as also showed by Bhumkar and Pokharkar[26]. However, in chitosan nanoparticles spectra (Fig. 2C) there is a strong presence of amine e amide bands of chitosan, but TPP bands (1155 cm^{-1} and 1554 cm^{-1}) are not so evident.

The nanoparticle size, concentration and molecular weight of chitosan can influence the antifungal activity of ChNP [27]. Peña, Sánchez & Calahorra[28] recommended the use of low molecular weight chitosan at concentrations higher than 1.0 mg/mL for assure a fungicidal activity and not only fungiostatic action. However, in the present study the $MIC_{83\%}$ of ChNP for the tested strains was 30.08 µg/mL, except for *C. albicans* CBS 562 that was 60.15 µg/mL. The MFC/MIC ratio reflected the fungicidal activity of ChNP for all the tested strains with lower concentration than 1.0 mg/mL. Furthermore, the MIC values were lower than that found by Ing et al.[2], which showed that the MIC of ChNP (with low molecular weight chitosan) against *C. albicans* was 250–860 µg/mL.

The time kill assay determined the influence of the exposure time of solutions on the cell death process. The concentration of 120.31 µg/mL (CIM x 4) was able to inhibit all fungal cell growth in a period of 4 h incubation (Fig. 6), demonstrating fungicidal activity similar to NaOCl. This satisfactory fungicidal effect of chitosan found, in concentration much lower than 1 mg/mL, probably occurred due to the nanoparticles formation of chitosan.

Qi et al.[21] demonstrated that chitosan nanoparticles exhibited higher antimicrobial activity than other chitosan derivates due to the special characters of the nanoparticles, such as the small size, the capacity to compact particle and the high surface charge. It is related that occurs the adsorption of chitosan onto cell walls, leading to membrane weakening, disruption and cell leakage[1,29,30,31,32,33]. The inhibition mechanism of chitosan nanoparticles against *C. albicans* was related to their capacity to diffuse into cells and to inhibit the DNA or RNA synthesis, subsequently causing a direct cell death[2].

The fungal micromorphology test was important to complement the fungicidal activity, because the morphological changes of cells are one of several factors that is associated with microorganism pathogenicity and virulence. The formation of hyphae and pseudohyphae is related to pathogenicity factors expressed by *C. albicans* because these structures cause epithelial tissue invasion and represent a barrier to phagocytosis[34]. At all concentrations tested in this study, ChNP reduced the formation of hyphae and pseudohyphae and provided a total inhibition of chlamydoconidia. Thus, there was a maintenance of fungal cells in the form of blascoconidia, which is considered the less virulent form.

Biofilm is considered as a dense layer of complex microbial communities embedded in a polymeric matrix that promote mutually beneficial interactions among microorganisms and protect them from diffusion and action of antimicrobial agents[35]. The adhesion of *Candida* spp. to the surface of acrylic resin is usually the first step in the colonization of tissues that are exposed to surface denture, and tends to form a biofilm if the surface is not clean[36]. Sodium hypoclorite 1% is one of major solutions used to disinfect denture base resins[11,13,37,38].

In the present study, the results of inhibitory effects of ChNP on the initial adherence, formation and reduction of *C. albicans* biofilm showed that the highest concentration tested (MIC x 4) was the most effective, as also related by other studies[2,6]. The ChNP showed positive results on the initial *C. albicans* adherence, since ChNP at MIC x 4 had % I similar to NaOCl, showing that ChNP is able to disaggregate the fungal adhesion and to interfere on biofilm formation. This fact was confirmed because the contact time of solutions with biofilm cells influenced the % inhibition. After 1 min contact time (G2) only ChNP at MIC x 4 showed higher % I on biofilm formation (51.1%) than NaOCl. After 8 h (G3) all concentrations of ChNP showed superior % I (50.2 to 57.8%) compared to NaOCl (Table 2). The comparison with other studies becomes difficult because there are investigations about ChNP only against *Candida* planktonic cells[2]. However, these results can show that

besides the concentration of ChNP, the contact time with the biofilm also influenced the CHNP antifungal activity against *C. albicans* biofilm and the ChNP solutions investigated have similar or superior anti-biofilm activity compared to NaOCl.

Chitosan has capacity to disrupt a preformed biofilm, not only by reducing yeast metabolic activity but also by promoting disaggregation of biofilm extracellular matrix[6]. However, there was no data in literature about the inhibition potential of ChNP on *C. albicans* mature biofilm using contact times and frequency of application of ChNP simulating the clinical application (1 min or 8 h). The present study showed that during 72 h *C. albicans* mature biofilm ChNP was effective to inhibit 52.2 to 66.1% of fungal growth after 3 applications of 1 min (G4) or 1 application of 8 h (G6), regardless of its concentration (Table 3). Moreover, ChNP with 1 application of 8 h had superior % I than NaOCl.

However, for 96 h *C. albicans* biofilm, only ChNP at MIC x 4 concentration was effective to inhibit mature biofilm, even with the increase of frequency of application and contact time (6 x 1 min - G5 or 2 x 8 h - G7) (Table 3). In addition, it also was observed that ChNP reduced its antifungal activity (36.6 % I) and NaOCl had superior %I (49.8%). These results suggest that for inhibition of mature biofilm (superior to 96 h) it would be interesting to increase the concentration of ChNP to find an effective antifungal activity.

As chitosan nanoparticles can penetrate and diffuse inside the biofilm and into yeast nuclei, causing its binding to DNA and inhibition of RNA synthesis, alterations occur into the cellular metabolism and promote cellular stress that causes biofilm disruption[39]. This fact could explain the superior results found for ChNP compared to NaOCl. The sodium hypochlorite acts in the cytoplasmic membrane integrity by irreversible enzymatic inhibition due to its high pH, causing biosynthetic alterations in the cell metabolism and the destruction of phospholipids due to the lipidic peroxidation[40].

Although the results of biofilm formation on acrylic resin evaluated the effect of ChNP and NaOCl against multi-species *Candida* biofilm, they were similar to that found in G6 and G7 with 8h application of solutions. After 48 h biofilm formation, ChNP at MIC x 4 and NaOCl reduced significantly the fungal colonization on acrylic resin (Fig. 8). After 5 days, NaOCl was more effective to reducing biofilm growth than ChNP (Fig. 9). In our study, 1% NaOCl was initially used, but the final concentration in wells was 2,500 µg/mL (CIM x 4), a superior concentration compared to ChNP. Even so, ChNP at 120.31 µg/mL was effective to reduce almost 50% UFC/mL *Candida* spp. colonized on acrylic resin surface. Acosta et al.[41] showed that NaOCl 1% in a cycle of 10 min per day for 7 days of disinfection, reduced *C. albicans* adhesion in approximately 60%, and in 30 days of

disinfection cycle, the number of adherent cells reduced in 80%[41]. However, others studies demonstrated that NaOCl was effective as denture disinfectant, reducing the adhesion of *Candida albicans* cells on denture acrylic, but other solutions were more effective in denture disinfection than NaOCl[11,42].

When disinfectant solutions are used to aid the cleaning of denture, their choice should be made with regard to its effectiveness in inactivating microorganisms without any adverse effects on the denture materials[38]. In the present study, ChNP did not cause alteration on roughness surface of acrylic resin after 48h or 5 days biofilm formation, but it reduced VHN surface. The surface roughness of acrylic resin has been shown to greatly influence adhesion and biofilm formation of *Candida* spp., with more roughened surfaces retaining more microorganisms and increasing the likelihood of microorganisms remained on the surface after the prosthesis has been cleaned[11,38]. These factors also are associated with more degradation of material surface[11]. The increase of Ra values found after NaOCl application and biofilm formation can be considered satisfactory, since the surface roughness value of heat-polymerized resins can range from 0.03 µm to 0.75µm[43].

However, NaOCl group had higher Ra values compared to ChNP groups and showed the lowest VHN values, provoking a surface degradation more accentuated. These results are similar to those of some studies[36,37,38] which showed that after immersion in NaOCl there was significant change in Ra and VHN values of acrylic resin. Probably, NaOCl components may penetrate into the denture base resin and result in softening of its surface[37]. Acrylic resins can absorb water molecules, which also act as plasticizers and affecting mechanical surface properties of denture base[38]. ChNP groups also showed reduction on VHN values after solution's application and biofilm formation (Table 5). The pH of 5.5 of ChNP solutions may have contributed for the softening of acrylic surface. However, the acidic pH of chitosan is necessary, because that the antimicrobial activity of chitosan and its derivatives is exhibited only when the pH is below the respective pKa, the value at which the soluble molecule could be disassociated as ions in solution[1].

These results demonstrated a good potential for application of ChNP for denture disinfection with daily 8h application. Innovative information about ChNP was demonstrated in this study about the antifungal activity and the anti-biofilm activity of ChNP on acrylic resin surface with positive action on the fungal micromorphology. However, this solution is still experimental and further researches are necessary to assess the stabilization of nanoparticles into solution and its antifungal activity on resistant clinical isolates.

5. CONCLUSION

The experimental chitosan nanoparticles solution synthetized in this study showed a good potential of application for denture disinfection, since ChNP had antifungal activity against planktonic *C. albicans*, *C. tropicallis* and *C. krusei*, inhibition capacity on *C. albicans* initial adherence and mature biofilm and anti-biofilm activity against multi-species biofilm formation on acrylic resin, causing minimal changes on its surface.

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2 CONSIDERAÇÕES GERAIS

O presente trabalho trata-se de um estudo inovador principalmente porque avalia a ação da solução de nanopartícula de quitosana (ChNP) sobre a micromorfologia fúngica, como também sobre o biofilme multi-espécie de Candida spp. na superfície de resina acrílica, além de sua ação nas propriedades de superfície da resina acrílica.

Os resultados demonstraram atividade da ChNP sobre Candida spp. demonstrando uma inibição de 83,3% das cepas estudadas, e com concentração CIM abaixo dos estudos encontrados na literatura. Entretanto, o teste da microdiluição não fornece subsídios da atividade antifúngica da ChNP quanto ao seu efeito nas células fúngicas em função do tempo, como também no que diz respeito à patogenicidade da morfologia destas células. Sendo assim, outros dois testes iniciais foram realizados para complementar esses dados. O primeiro foi o teste da cinética de crescimento microbiano (*Time Kill assays*), o qual é uma variável importante para avaliar a atividade fungistática ou fungicida dos compostos e determinar a influência do tempo de exposição no processo de morte celular. E o segundo, foi o teste da alteração na micromorfologia das células, já que estas alterações são associadas à patogenicidade do micro-organismo. Neste teste pode ser observado a formação de hifas e pseudohifas relacionadas aos fatores de patogenicidade expressos pela cepa fúngica, uma vez que estas estruturas representam uma barreira à fagocitose e permitem adesão das leveduras sobre o tecido epitelial. O presente estudo foi o primeiro estudo que demonstrou a atividade da ChNP na mcromorfologia fúngica, o que tornou inclusive difícil a comparação dos resultados com a literatura, e demonstrou redução da formação de hifas e pseudohifas e proporcionou uma inibição total de clamidoconidios. Esses dados foram encontrados com maior significância em uma concentração mais elevada da ChNP (120,31 µg /mL - CIM x 4). Entretanto, esta concentração ainda apresentou-se menor do que a encontrada em outros estudos.

Tentando evoluir os dados para uma aplicação clínica da ChNP foi realizado a avaliação sobre biofilme de Candida spp. Mesmo que alguns estudos recentes tenham revelado uma atividade anti-biofilme significativa da solução sobre vários micro-organismos, incluindo *C. albicans*, pouco se sabe sobre a atividade da ChNP no processo de ciclos de imersão diário e biofilmes maduros. Por isso, optou-se por um estudo inicial mono-espécie com a utilização de cristal de violeta e leitura em Leitor de microplacas onde foi avaliado a aplicação da substancia por dois tempos: 1 min (simulando a imersão da prótese na solução enquanto o paciente realiza sua higiene diária, imaginando um paciente que faz

escovação/higienização 3x ao dia), como também por 8h (simulando a imersão da prótese não solução no período da noite, quando o paciente remove a mesma por um período mais prolongado). Sendo assim, estudou-se duas aplicações clínicas da mesma solução.

Tentando transpor ainda mais essa linha entre o clínico e o laboratorial, o efeito da ChNP na atividade anti-biofilme foi analisada também sobre o biofilme multi-espécie (*C. albicans*, *C. tropicallis* and *C. krusei*) desenvolvido na superfície da resina acrílica e seu efeito nas propriedades de superfície da mesma. Este estudo foi conduzido afim de elucidar dúvidas acerca de sua aplicação sobre um biofilme mais estruturado biologicamente sobre o material mais utilizado em prótese, como também se sua aplicação poderia causar alterações sobre a resina, o que o tornaria inviável para aplicação clínica.

Embora o teste em resina acrílica tenha avaliado o efeito do ChNP contra um biofilme multi-espécies de Candida, os resultados da formação de biofilme foram semelhantes aos encontrados no G6 e G7 do teste em placas de microdiluição com aplicação da solução por 8h.

A ação da ChNP foi comparada, em todos os testes realizados, com o hipoclorito de sódio, solução essa com eficácia comprovada na literatura sobre cepas fúngicas, bem como na sua aplicação como desinfetante de prótese pelos pacientes no dia a dia. Entretanto, apesar da sua eficiência como um desinfetante, o hipoclorito de sódio possui algumas desvantagens, incluindo a sua atividade corrosiva sobre as superfícies metálicas e acrílicas, o efeito irritante sobre a pele e outras células. Com relação aos resultados das propriedades superficiais (rugosidade e microdureza), a ChNP mostrou-se satisfatória com mínima alteração na microdureza, já que somente a solução de hipoclorito de sódio apresentou aumento na rugosidade significante no período de 5 dias de formação de biofilme.

Considerando que a Candida spp. é o principal agente etiológico da candidíase oral, mediante os resultados encontrados a ChNP apresenta um potencial de aplicação para uso clínico, o que possibilita sua utilização na desinfecção da prótese como uma abordagem terapêutica preventiva. Entretanto, outras investigações devem ser feitas como avaliar a estabilidade da solução, estudos clínicos *in situ* ou *in vivo.*

3 CONCLUSÃO

Diante dos resultados obtidos pode-se concluir que:

1. A espectroscopia de infravermelho comprovou a presença de grupos característicos da quitosana e TPP na solução sintetizada, podendo representar um indício de que a superfície da nanopartícula é composta basicamente de quitosana e que o TPP encontra-se em seu interior;
2. A MET mostrou que o método de geleificação iônica foi eficaz na síntese de nanopartículas de quitosana, apresentando formato arredondado e regular, com tamanhos médios de aproximadamente 0,05 µm (50 nm);
3. A $CIM_{83.3\%}$ da ChNP capaz de inibir 83,3% das cepas estudadas foi de 30.08 µg/mL, exceto para *C. albicans* CBS 562 que apresentou valor de CIM de 60.15 µg/mL. A relação CFM/CIM revelou uma atividade fungicida do composto estudado para todas as cepas.
4. A concentração de 120,31 µg /mL (CIM x 4) de ChNP foi capaz de inibir o total crescimento celular fúngico em um período de 4 horas de incubação e mostrou inibição de pseudohifas e clamidoconídeos, apresentando somente a micromorfologia de blastoconídeos.
5. Nos ensaios de aderência, formação e redução do biofilme, a maior concentração testada da ChNP (CIM x 4) foi a mais eficaz na inibição de *C. albicans.*
6. A solução ChNP na CIM x 4 demonstrou maior redução de UFC de Candida spp. sobre a superfície de resina acrílica, após 48h de biofilme. Após 5 dias de biofilme, todas as concentrações de ChNP reduziram UFC de Candida spp., entretanto hipoclorito de sódio apresentou maior redução. Apenas a solução de hipoclorito de sódio apresentou aumento de Ra significante no período de 5 dias, enquanto que para VHN todos os grupos apresentaram diminuição significante nos valores VHN.

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