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Plínio Mendes Senna
Cirurgião-Dentista

Influência da extensão da área colonizada por biofilme de *Candida albicans* na efetividade da desinfecção de próteses por energia de micro-ondas

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Mestre em Clínica Odontológica, Área de Prótese Dental.

Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

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“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original.”

Albert Einstein

Resumo

A candidose oral é a infecção fúngica mais comum diagnosticada em humanos. Em indivíduos com sistema imunológico comprometido, pode disseminar-se sistemicamente causando um quadro denominado de candidemia, o qual está relacionado com alta mortalidade e aumento do tempo de permanência hospitalar. A candidose é frequentemente associada ao uso de próteses removíveis, apresentando-se como uma inflamação da região recoberta da mucosa, o que demanda a necessidade de controle microbiológico no biofilme patogênico acumulado na prótese para prevenção da candidose oral. Assim, o objetivo deste estudo foi verificar se a extensão da área da prótese removível colonizada com biofilme de *Candida albicans* influencia o processo de esterilização por energia de micro-ondas. Cento e vinte próteses totais superiores estéreis tiveram áreas distintas de 2,35cm^2 ou 5,50cm^2 colonizados com biofilme de *Candida albicans* de 72 horas. Cada prótese foi imersa em 200 mL de água destilada estéril e irradiada com potências de 450, 630 ou 900 W por diferentes tempos (1,2 e 3 min) até que não fosse detectada a presença de células fúngicas viáveis (n=6). Também a temperatura final da água foi aferida imediatamente após a irradiação para verificar sua influência neste processo. Os resultados apresentaram diferença estatística significante para a extensão da área colonizada ($p<0,001$), sendo que a área de maior extensão demandou um maior tempo de irradiação para esterilização. Nas próteses totais com a maior área colonizada e irradiadas por 3 minutos, independente da potência não foi detectado crescimento fúngico, sendo significantemente diferente dos demais tempos ($p<0,001$). As próteses com menor extensão de área colonizada apresentaram-se estéreis após 1 minuto a 900 W e 2 minutos para 450 e 630 W. Houve correlação positiva entre a temperatura final da água e a eficácia da esterilização (r=0, 6170). Considerando as limitações deste estudo, é possível concluir que quanto menor a extensão da área colonizada por biofilme menor é o tempo de irradiação por micro-ondas requerido para a esterilização.

Palavras-chave – *Candida albicans*, candidose, prótese, desinfecção, micro-ondas.

Abstract

Oral candidosis is the most common fungal infection diagnosed in humans. In subjects with compromised immune system, it can disseminate to candidemia, which is related to high mortality and more hospital time consumption. Candidosis is commonly related to denture wearers, characterized by an inflammation of the coverage portion of mucosa, demanding the necessity of microbiologic control of the biofilm accumulated on denture surface for prevention of oral candidosis. Therefore, the aim of this study was to evaluate the influence of *Candida albicans* biofilm coverage area on the effectiveness of microwave disinfection. One hundred twenty sterile dentures have two distinct areas, 2.35 or 5.50cm^2, coverage by 72 hours *Candida albicans* biofilm. Each denture was immersed in 200 mL of sterile distilled water and was irradiated by 450, 630 or 900 W and different exposure times (1, 2 e 3 min) until no viable cells were detected (n=6). The final temperature was also measured to verify its influence on this process. The results showed statistical difference between the biofilm coverage area ($p<0,001$), in which a higher coverage demands more exposure time for sterilization. The dentures with higher biofilm coverage and irradiated for 3 minutes, independently of power set, showed no fungal growth, this exposure time was statistically different from 1 and 2 min ($p<0,001$). Dentures with lower biofilm coverage were sterilized after 1 min at 900 W and 2 min for 450 and 630 W. There was a positive correlation between the final water temperature and the effectiveness of sterilization (r=0, 6170). Considering the limitations of this study, it is possible to conclude that lower biofilm coverage demands a lower microwave exposure time for sterilization achievement.

Keywords: *Candida albicans*, candidiasis, denture, disinfection, microwave.

Sumário

1. Introdução

A resina a base de poli(metilmetacrilato) (PMMA), tem sido utilizada por longo tempo como material odontológico restaurador, principalmente em próteses removíveis. Apesar do seu sucesso clínico, um dos principais problemas envolvendo as reabilitações com este material é o acúmulo de biofilme sobre a sua superfície (1).

Biofilmes consistem de uma comunidade composta por vários micro-organismos protegidos por uma matriz extracelular que pode se desenvolver sobre qualquer superfície (2). A cavidade bucal apresenta biofilmes sobre diferentes substratos e diferentes espécies orais e não-orais (3, 4), incluindo bactérias de gêneros como: *Staphylococcus* spp., *Lactobacillus* spp., *Pseudomonas* spp., *Enterobacter* spp. *Streptococcus* spp. e *Actinomices* spp.. Estes micro-organismos estão relacionados com doenças locais como cárie e doença periodontal (5), entretanto, em pacientes com desequilíbrio do sistema imunológico, podem estar relacionadas à doenças sistêmicas como infecção do trato urinário, conjuntivite, pneumonia e meningite (4).

A presença destes biofilmes na superfície de próteses removíveis gera uma via potencial de contaminação cruzada entre o paciente, dentista e técnico de laboratório, quando há a necessidade de intervenção de manutenção nestas próteses. Powell *et al.* (6) observaram que 67% de todo o material recebido pelos laboratórios de prótese odontológica estavam contaminados com alguma espécie patogênica, evidenciando a necessidade de desinfecção destas próteses antes de serem enviadas ao laboratório. Também foi verificada a presença de patógenos no material e instrumental laboratorial, assim as próteses também deveriam ser desinfetadas antes de serem entregues ao paciente (7). Portanto, para controle de infecção cruzada causada pelas próteses removíveis, há a necessidade de dois ciclos de desinfecção, um antes de enviar ao laboratório e outro antes da instalação no paciente.

Além destes micro-organismos que compõem os biofilmes sobre a resina de PMMA, os fungos pertencentes ao gênero da *Candida* spp. tomam bastante importância na atualidade, já que a epidemiologia das infecções fúngicas modificou-se nos últimos 20 anos (8). Percebe-se um aumento de sua incidência e expansão da população de risco pela

presença de algumas condições médicas como: transplantes, cânceres, terapia imunosupressiva, AIDS, parto prematuro, idade avançada e grandes cirurgias (8). A candidose e mais recentemente a candidemia não estão apenas relacionadas com alta mortalidade (30-40%), mas também com o aumento do tempo de permanência hospitalar e os dos custos associados (8-10).

A infecção oral fúngica mais comum diagnosticada em humanos é a candidose associada ao uso de próteses, também chamada de estomatite protética (11), apresentando-se como uma inflamação da mucosa, cuja prevalência chega a 67% nos usuários de próteses removíveis (12-14). Esta patologia tem a *Candida albicans* associada como o seu principal agente etiológico, pela sua alta virulência e eficiência em aderir e colonizar superfícies resina de PMMA. Um dos fatores responsáveis por esta eficiência é denominado dimorfismo celular, onde, dependendo das condições de crescimento, há a conversão morfo-genética de alguns fungos de célula germinativa ou levedura para a forma de hifa ou pseudo-hifa (15, 16). Durante o processo de colonização, algumas espécies de *Candida* convertem sua forma de levedura para hifa e durante sua expansão ocorre o fenômeno conhecido como tigmotropismo, que é a capacidade de reconhecimento de uma superfície pelo contato (17-20). Essa característica no padrão de colonização faz com que a hifa se desenvolva e se aloje no interior das irregularidades presentes na superfície de resina de poli(metilmetacrilato) (21). Na forma de hifa, que acontece favoravelmente em meios ricos em proteínas (15, 17), a secreção de enzimas hidrolíticas (fosfolipases e aspartil-protease) é o fator responsável pela irritação da parede celular epitelial (15, 16, 22). Isto promove o desencadeamento de um processo inflamatório que se caracteriza por um aspecto edemaciado e eritematoso, em parte ou em toda a mucosa do palato (23).

Esta patologia é comumente tratada com a utilização de nistatina como um agente antifúngico tópico, por se tratar de uma droga que gera menos efeitos colaterais devido a sua posologia fracionada (400.000 a 600.000 UI, 4 vezes ao dia durante 14 dias, orientando o paciente a deglutir a suspensão após o bochecho bucal) (24). O sucesso da terapia é observado após estes 14 dias com a eliminação de leveduras e a redução dos sinais clínicos da candidose. Entretanto, 40 dias após a suspensão do tratamento, a colonização por leveduras, e o aspecto edemaciado, atinge índices semelhantes aos encontrados

anteriormente ao tratamento (25). Este fenômeno de reinfecção também pode favorecer a seleção de cepas de *Candida albicans* anti-fúngico resistentes (26), apesar de ainda ser rara a prevalência de cepas resistentes ao fluconazol em candidemias (27).

Além do estabelecimento de espécies resistentes aos antifúngicos, outro fator está relacionado com a freqüente reincidência desta doença. O biofilme acumulado na prótese removível atua como um reservatório de *Candida* spp. (23), e este vai promover a recontaminação da mucosa palatina do usuário. E esta reinfecção será, a cada evento, mais virulenta e antifúngica-resistente (28, 29). Assim, o tratamento da candidose associada ao uso de próteses removíveis deve ser direcionado primeiramente para o controle do biofilme acumulado sobre a prótese (30).

O passo inicial no tratamento poderia ser a paralisação do uso da prótese e interrupção do contato entre o biofilme e a mucosa, pelo menos sua remoção noturna, mas esta opção interfere diretamente no aspecto psicossocial do paciente (31), além de apresentar alta reincidência clínica da patologia (7). Assim, a escovação mecânica com dentifrício é o método mais amplamente utilizado para a limpeza da prótese e, consequente prevenção do acúmulo de biofilme (32), mas pacientes com destreza manual deficiente ou acuidade visual reduzida (33) apresentam limitada capacidade de higienização das próteses (32).

Logo, métodos complementares se fazem necessários, dentre eles incluem-se a desinfecção por meios químicos, como a imersão em glutaraldeído alcalino a 2% por 10 minutos, imersão em hipoclorito de sódio a 1% por 10 a 30 minutos e imersão em formaldeído a 3% por 30 minutos. No entanto, problemas têm sido descritos com o uso destas substâncias como a alteração de cor da resina e a corrosão de componentes metálicos sob a ação do hipoclorito (34, 35). Além disso, desinfetantes a base de álcool não devem ser utilizados em próteses confeccionadas com resina de poli(metilmetacrilato) que não contenham componentes para formação de ligações cruzadas entre as cadeia poliméricas (cross-link), pois o mesmo altera a superfície reduzindo a sua resistência à flexão (36). Também deve ser considerada a citotoxicidade (37) e o potencial mutagênico e carcinogênico (38, 39) do agente desinfetante, uma vez que podem impregnar-se pelos

poros da resina, não sendo possível a sua completa remoção apenas com a lavagem da prótese em água corrente.

A desinfecção por meio de energia de micro-ondas é uma alternativa para estes agentes químicos. Vários estudos já demonstraram a eficiência do aparelho de micro-ondas doméstico na esterilização de material laboratorial (40) e instrumental odontológico (41) e para desinfecção de lentes de contato (42), apesar da maneira pela qual a radiação de micro-ondas age no nível celular ainda não seja completamente entendida (43).

A eficiência da energia de micro-ondas contra os micro-organismos bucais já foi estudada (44-52), no entanto os diversos regimes de irradiação utilizados dificultam comparações entre os seus resultados. Com um ciclo utilizando potência máxima de um micro-ondas e tempo de exposição de 5 minutos, Dixon *et al.* (45) demonstraram a influência do meio onde a prótese é irradiada, seco ou molhado, no processo de desinfecção. Quando suas amostras foram irradiadas submersas em água destilada houve esterilização para o fungo *Candida albicans*. No entanto, outras amostras irradiadas em ambiente seco mostraram que ainda há remanescente fúngico.

Outra variável é a potência utilizada e o tempo de exposição. Rohrer e Bular (49) obtiveram resultados satisfatórios utilizando 720 W de potência com um tempo de exposição de 10 minutos para um grupo de 4 bactérias aeróbias e um fungo. Webb *et al.* (52) demonstrou que 350W por 6 minutos é um método eficiente para esterilização de *Candida albicans* e *Streptococcus gordonii*. Banting e Hill (44) mostraram que um ciclo de 850 W durante um 1 minuto é um método eficiente de desinfecção da prótese, adjunto à medicação antifúngica para o tratamento de estomatite, sendo este ciclo realizado em ambiente seco.

Silva *et al.* (51), usando corpo-de-prova submerso em água destilada, e ciclo de 650 W de potência com 6 minutos de exposição, mostrou a eficiência sobre próteses removíveis colonizadas com três bactérias (*Pseudomonas aeruginosa*, *Streptococcus aureus* e *Bacillus subtilis*) e para a *Candida albicans*. Houve esterilização para *S. aureus* (gram-positivos) e *C. albicans* (fungo) e desinfecção para *P. aeruginosa* (gram-negativa) e *B. subtilis* (esporo bacteriano). Pavarina *et al.* (53, 54) demonstraram que dois e sete ciclos de 650 W/6

minutos não afetam a resistência à flexão de reembasadores rígidos nem a dureza dos dentes acrílicos.

Ainda ciclos de 650 W por 5 minutos foram propostos em ambiente seco, mostrando redução do número de bactérias. No entanto, para este ciclo não há estudo sobre seus efeitos sobre a resina. Já para ciclos de 500 W com até 15 minutos de exposição em estado seco, a resina não apresenta alteração dimensional significativa clinicamente (menor que 0,03%), nem alteração da resistência à flexão (55). Webb *et al*. (56) relatam ainda que uma potência menor é desejável para se evitar efeitos adversos sobre a resina de PMMA.

De acordo com a revisão da literatura, estudos envolvendo o processo de esterilização por energia de micro-ondas, simulando próteses com diferentes áreas cobertas por biofilmes maduros não foram encontrados e, considerando a importância clínica de se poder estabelecer protocolos para desinfecção de próteses considerando a área colonizada, o objetivo deste estudo foi avaliar a influência da extensão da área contaminada na eficiência do processo de desinfecção por energia de micro-ondas.

2. Capítulo

Influence of *Candida albicans* biofilm coverage area on denture disinfection by microwave energy

Plínio Mendes Senna[1]

Wander José da Silva[1]

Altair Antoninha Del Bel Cury[1]

[1]Department of Prosthodontics and Periodontology,

Piracicaba Dental School, State University of Campinas,

Campinas, São Paulo, Brazil.

Corresponding author:

Altair A. Del Bel Cury

Piracicaba Dental School, State University of Campinas

Department of Prosthodontic and Periodontology

Piracicaba, SP, Brazil.

Phone: 55 19 21065294

E-mail: altcury@fop.unicamp.br

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Abstract

Objective: This study evaluated the influence of *Candida albicans* biofilm coverage area on dentures disinfection by microwave energy.

Materials and methods: One hundred and twenty sterile maxillary dentures were submitted to distinct biofilm coverage areas of 2.35 cm^2 (small area) or 5.50 cm^2 (large area) for 72 h *C. albicans biofilm* colonization. Each denture was immersed in 200 mL distilled water and individually irradiated at 450, 630 or 900 W of power for different times (1, 2 or 3 min) until no viable cells were detected (n=6). The final temperature was measured immediately after irradiation to verify its influence on this sterilization process. Data were analyzed by ANOVA, followed by Tukey´s test with a level of significance fixed at 5%.

Results: Dentures with larger biofilm coverage demanded a higher irradiation exposure for sterilization ($p < 0.0001$), irrespective of power setting, and in this time no yeast growth was detected. Dentures with small biofilm coverage area were sterilized after one minute at 900 W and 2 min at 450 or 630 W. A positive correlation was found between water temperature and sterilization effectiveness (r=0.6170).

Conclusion: It was possible to conclude that biofilm coverage area had influence on microwave energy sterilization process and that dentures with larger biofilm coverage area required higher irradiation exposure to achieve sterilization.

Keywords: *Candida albicans*, candidosis, denture, sterilization, microwave energy

Introduction

The presence of *Candida* spp. biofilm on dentures can lead to chronic erythematous candidosis (CEC), the most prevalent form of oral candidosis, affecting over 65% of denture wearers[1]. Oral candidosis is often restricted to local mucosa reaction, however immunocompromised subjects may present aggravated systemic infection known as candidemia, which can cause a 40% mortality rate or a longer hospitalization time[3, 4], leading to a higher cost to the health system[2].

The main etiological factor for candidosis is the high level colonization of pathogenic microorganisms on denture surfaces[3]. The fungus *Candida albicans* is the main etiological factor, due to its high virulence and ability of surface adhesion and colonization on poly(methylmetacrylate) resin (PMMA) surfaces, forming structured communities known as biofilms[3].

Systemic antifungal therapy is usually not sufficient to treat candidosis, because of re-infection commonly caused by the use of a contaminated denture. Therefore, the treatment must primarily be directed towards the denture and secondarily to the mucosa[4]. Thus, an initial measure could be to interrupt the use of dentures colonized by *C. albicans*, however this action has high impact on patient's psychosocial aspects[5].

Mechanical brushing is effective in preventing fungal adhesion by removing food debris and microorganisms. However, some patients do not have the visual acuity or the manual dexterity to perform denture hygiene properly, which leads to biofilm accumulation[6] and the need for auxiliary cleaning methods. Among these methods, immersion in chemical disinfectants such as sodium hypochlorite, alkaline glutaraldehyde

and formaldehyde are commonly used and they are very effective. However, these cleaners may cause some adverse effects on PMMA resin, such as a reduction in surface hardness[7] and flexural strength[8], and may cause bleaching[7, 9]; moreover, the clinical recurrence of candidosis is common after their use is interrupted[10].

To avoid these adverse effects, microwave energy irradiation has been proposed as fast and clean method for disinfecting dentures and in the treatment of denture stomatitis[11] and in the prevention of clinical cross infection[12]. Although its action mechanism remains unclear, its effectiveness has been demonstrated in vitro against planktonic cells of *C. albicans*[13] and young 24-hours biofilms[14, 15] when irradiated by microwaves. As regards the immersion medium, its maximum effectiveness is achieved when the substrate colonized by microorganisms is irradiated immersed in water[16].

The choice of an ideal irradiation regimen should consider factors such as the nature of the substrate, quantity and location of microorganisms, and power and time settings. Moreover, the organization of the extracellular structure of polysaccharides present in mature *C. albicans* biofilms, which promotes antifungal resistance[17], may influence the biofilm susceptibility to microwave energy. Thus, the aim of this study was to verify the effectiveness of microwave disinfection in the sterilization of dentures with different *C. albicans* biofilm coverage areas.

Materials and methods

Experimental design

In this in vitro study, the microwave irradiation assay was randomized and the microbiology analysis was blind. PMMA resin discs (10 x 1.5 mm) colonized by 72-h *C. albicans* biofilm were placed in niches prepared on the palatal surface of sterile dentures. This procedure was done in order to mimic the condition of a denture with a large or small area covered by biofilm. The group with smallest biofilm coverage had a colonized area of 2.35cm^2 (3 discs) and the highest, 5.50cm^2 (7 discs) of biofilm coverage. The denture with the colonized discs was immersed in 200 mL distilled water and placed in the center of a domestic microwave oven. Then each denture (n=6) was irradiated by 450, 630 or 900 W for 1, 2 or 3 min, until no viable cells were detected by yeast count analysis, determining absence or presence of sterilization. After the irradiation time, the final temperature was immediately measured to verify its influence on this process.

Removable denture fabrication

A master cast was used to prepare denture replicas made of heat-polymerizing PMMA (Lucitone 550, Dentsply, USA). After finishing and polishing, the dentures were immersed in distilled water for 48 h at 23 ± 1,0 °C for residual monomer release [23]. Next, the dentures were ultrasonically cleaned (Thornton T 740; Thornton-Inpec Eletrônica LTDA, Vinhedo, Brazil) for 20 minutes and sterilized with ethylene oxide (ACECIL Comércio e Esterilização a Óxido de Etileno Ltda, Campinas, Brazil). These dentures had

niches on the palatal surface measuring 11mm in diameter x 1.5 mm deep to seat the contaminated discs during microwave irradiation.

PMMA resin disc fabrication

A metal mould was used to make PMMA heat-polymerized discs (Lucitone 550; Dentsply, USA) measuring 10 mm in diameter and 1.5 mm thick, in accordance with the manufacturer's instructions. The discs were finished and polished using progressively smoother aluminum oxide papers (grit 320, 400 and 600) in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil)[18] and immersed in distilled water for 48 h at room temperature (23 ± 1.0°C) for residual monomer release[19].

Next, the surface roughness (Ra) of these discs was measured using a profilometer (Surfcorder SE 1700 Kozaka Industry, Kozaka, Japan) with a precision of 0.01 μm, calibrated for a specimen length of 0.8 mm, 3.2 mm measurement range at 0.5 mm/s. Three readings were made for each specimen and a mean value was calculated[18]. A roughness of 3.3 ± 0.5 Ra was standardized, to ensure no influence of disc surface on fungal adhesion. After this, the PMMA discs were disinfected in an ultrasonic bath (model T740; Thornton-Inpec Eletrônica, Vinhedo, Brazil) for 20 min[20].

Saliva acquired pellicle formation

All specimens received a salivary pellicle coating prior to biofilm development in order to simulate the mouth conditions. Human whole saliva was collected from a single healthy volunteer, who had not used antibiotics, mouth rinses, or any other medication known to affect salivary composition and flow in the past 3 months, and who provided

written informed consent previously approved by the Local Ethics Committee. Stimulated saliva was collected and clarified by centrifugation at 10.000 g for 10 min at 4 °C. The supernatant was sterilized by 0.22 μm membrane filtration (TPP, Switzerland) and 1 mL of sterile saliva was placed into each well of a sterile 24-well microtiter plates with the PMMA disc specimens, which remained there for 30 min to form an acquired pellicle[18]. After this period, specimens were removed, washed twice with sterile PBS and immediately used in the biofilm development assay.

C. albicans biofilm assay

For analysis of the effectiveness of microwave disinfection, yeast strains of *C. albicans* (ATCC 90028) were aerobically cultured at 37 °C for 24 h on Sabouraud Dextrose Agar (SDA), and a loopful of growth was inoculated into Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI) supplemented with 50 mM glucose. After 18 to 20 h of incubation, cells were washed twice with PBS and suspended in YNB supplemented with 100 mM glucose. Standard *C. albicans* suspensions were prepared to a concentration of 10^7 cells/mL, adjusting optic density to 520 ηm wave length (Du 530 UV/visible spectrophotometer; Beckman Coulter Inc., Fullerton, USA)[21].

Biofilms were developed on PMMA disc surface microwave placed inside pre-sterilized flat-bottomed 24-well microtiter plates (TPP, Switzerland). Aliquots of 2.0 mL of standard cell suspensions of yeasts were transferred into each well with one disc and incubated for 90 min at 37 °C in an orbital shaker (Lab-line Incubator Shaker, Elliott Bay Laboratory Services, USA) at 75 rpm (adhesion phase). After the adhesion phase, the cell suspension was gently aspirated and each specimen was washed twice with PBS. For the

biofilm phase, 2.0 mL of freshly prepared YNB supplemented with 100 mM glucose was added to each well. The plates were incubated for 72 h at 37 °C at 75 rpm. After each 24 h of incubation, medium was aspirated and specimens were washed with PBS, followed by the addition of fresh 2.0 mL medium. After 72 h, the PMMA discs were randomly distributed among the denture niches and submitted to the microwave disinfection test.

Microwave Disinfection Test

The microwave disinfection test was performed in a 900 W domestic oven (AW-42 model; Continental, Manaus, Brazil). It was used the 630 W as an approximation to a standard power set[12, 22], a lower one (450 W) and oven's full power (900 W). Moreover, a small drop in output power could be verified after the first use of the microwave oven, therefore, before any further use, one calibration setting was performed to warm up the oven, irradiating 1 L of distilled water for 2 min at full power[23].

The dentures were immersed in 200 mL of distilled water[24] at room temperature (23 ± 1.0 °C) inside a glass Beaker, and placed in the center of the spin plate and individually irradiated at the power and for the proper time for each group. After exposure to irradiation, the denture was immediately removed and the final temperature of the mixed water was measured using a digital thermometer.

Next, the PMMA discs were transferred individually to a plastic tube containing 4 mL PBS and sonicated for 30 s for disaggregation of the biofilm. Then, 1 mL of this suspension was serially diluted and 3 drops of 20 μL for each dilution were seeded in SDA and incubated for 48 h at 37 °C to determine the remaining viable *C. albicans* cells.

Statistical Analysis

All analyses were performed using the SAS software (SAS Institute Inc., version 9.0, Cary, NC, USA) using a level of significance fixed at 5%. The normality of error distribution and the degree of non-constant variance were checked for the response variable (visible grow of viable cell) using the SAS/LAB package. The coverage area data were analyzed using one-way ANOVA and two-way ANOVA was used to analyze power and time variables. Tukey's HSD test was used as post ANOVA. The correlation between effectiveness of sterilization and temperature were analyzed by Pearson's correlation test.

Results

The biofilm coverage area was shown to have an influence on the microwave disinfection process ($p < 0.0001$). Dentures with larger biofilm coverage demanded longer exposure to microwave irradiation exposure when compared with the smaller coverage group (table 1).

On dentures with smaller coverage area, 900 W power produced sterilization after one minute of exposure, but when 450 W was used these dentures were sterilized after only 2 min ($p < 0.0001$) (table 1).

All dentures with larger biofilm coverage were sterilized after 3 min of exposure, irrespective of the power setting used (fig. 01). There was statistical difference among irradiation times ($p < 0.0001$), in which a progressive reduction in viable cells could be observed for the evaluated times.

The effectiveness of microwave disinfection showed a positive correlation with the temperature reached during the irradiation (r=0.61).

Discussion

The adhesion of microorganisms on denture surface, with the development of a polymeric matrix around colonies is an important factor of biofilms formation that can leads to candidosis. It is established that 650 W of microwave irradiation for 3 min is effective against microorganisms attached within 24 h[12-15, 25, 26], in which the extracellular polymeric matrix is not yet structuring the biofilm. In this study, it was evaluated the disinfection of dentures coverage by a mature biofilm of 72 h and the influence of the biofilm coverage area in this process of three different power sets. It was observed the sterilization of dentures with a biofilm coverage area until 5.50 cm^2 by 450 W microwave irradiation for 3 min.

The biofilm coverage area over denture surface is an important factor to be considered when microwave disinfection is proposed. In the present study, dentures with large biofilm coverage required 3 min of exposure to irradiation for sterilization, irrespective of the power setting used. However, 2 min of exposure was able to reduce the remaining viable cells by 98% in dentures with high coverage (fig. 01), exposure time that, despite of an expressive reduction, can favor new biofilm formation. The coverage area difference is related to different denture sizes or different levels of biofilm accumulation, what leads to different microwave susceptibility.

The use of microwave energy as a disinfection method can be explained by two distinct mechanisms. The first consider that the thermal aspects of medium heating promoted by microwave energy leads to microorganisms inactivation[27]. The other mechanism considers the cell inactivation by intracellular selective heating with membrane changes and internal destructuration[28].

The selective heating theory proposes that the cytoplasm is more susceptible to heat by microwave energy than the medium because of its higher ionic concentration, thus microwave irradiation is more effective than the conventional heating process. The magnetic field inside the microwave oven creates different electrical charges on microorganism membrane, leading to porous formation and membrane rupture, or destructuring of critical internal molecules as well[28]. These non-thermal effects increased the microorganism death rates, so they died at a lower temperature than in the conventional high temperatures treatments[29].

Nevertheless, some studies have shown that heating is the main factor for microorganism inactivation because no difference was found between conventional and microwave heating[30, 31]. A progressive cellular alteration is observed as a function of microwave energy exposure time[26, 32], however the effect on the biofilm complex is not reported in literature. Furthermore, the non-thermal effects would be the result of a lack of precision in measuring microorganism death in a temperature × time curve[27]. Despite of in the present study there was a positive correlation between the effectiveness and temperature, the exposure time seems to be more important than the final temperature when sterilization was perceived after 3 min (76 °C) and dentures after 2 min (91 °C) (table 1) wasn't sterilized.

Candida spp. cells may survive microwave energy when the substrate is irradiated in a dry state[16, 33], therefore, the water volume where dentures are immersed is important and it will be responsible for the thermal medium effects of cell inactivation. The microwave is not a direct source of heat, the electromagnetic waves are absorbed by resonance by polar molecules (water), promoting molecules excitation that reflects in heat production. Water promotes a homogenous medium heating, able to inactive microorganisms besides of destructuring of the extracellular complex of whole biofilm[34]. Therefore, the water volume is important to the final temperature and consequently for the thermal factor of this process. With a higher volume, lower it is going to be the final temperature, which may require higher exposure time to reach the cells killing temperature. In this study, 200 mL of distilled water was used because it is a sufficient volume to completely cover a denture[24] and it is capable of being reproduced in the clinical routine or in the patient's home. Moreover, 200 mL is suitable for protecting the microwave oven from the excess energy reflected inside the oven[33]. Also, the lowest possible power set used in microwave disinfection is important to avoid adverse effects on PMMA resin.

Three minutes of irradiation at 650 W is shown effective, however its effectiveness may be related to the fact that water starts to boil after 1.5 min[25]. Therefore, in addition to the thermal and non-thermal aspects, the movement of water bubbles can mechanically remove the microorganisms from de PMMA surface[26]. Although the boiling water is desirable for sterilization, its temperature could adversely affect the PMMA resin. Temperatures close to the glass transition temperature of PMMA (Tg 100.4°C) can modify the mechanical properties by the release of internal stress[35, 36]. Consequently, the lower irradiation regimen (lower exposure and lower power) able to sterilize with lower

temperature is desirable[4]. In this way, it was confirmed the effectiveness of a lower power set (450 W), showing efficient to sterilize *Candida albicans* mature biofilms (table 1), at a supposedly safer temperature for PMMA.

Therefore, with the results of the present study, it was shown that for different extension of biofilm coverage area the irradiation time needs to be adjusted, at a same power set, allowing the each denture wearer uses a personalized irradiation regimen for denture disinfection at domestic microwave oven.

Conclusion

Within the limitations of this study, was possible to conclude that biofilm coverage area had influence on microwave energy sterilization process and that dentures with larger biofilm coverage area required higher irradiation exposure to achieve sterilization.

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Table 1 Effectiveness of sterilization and final temperature after the microwave disinfection assay (mean ± s.d.); n=6.

Real power (W)	Time (min)	Coverage	Sterilization	Final temperature (°C)
450 a	1 A	small A	-	44.33 ± 0.58
		large B	-	
	2 B	small A	+	62.45 ± 1.23
		large B	-	
	3 C	small A	+	76.97 ± 1.96
		large B	+	
630 ab	1 A	small A	-	50.08 ± 0.50
		large B	-	
	2 B	small A	+	72.22 ± 0.84
		large B	-	
	3 C	small A	+	90.73 ± 1.83
		large B	+	
900 b	1 A	small A	+	58.25 ± 1.96
		large B	-	
	2 B	small A	+	91.45 ± 2.29
		large B	-	
	3 C	small A	+	98.33 ± 1.91
		large B	+	

+. presence of sterilization; -.absence of sterilization

Different letters show significant statistic difference. Capital letters show $p<0.001$ and lower case $p<0.05$.

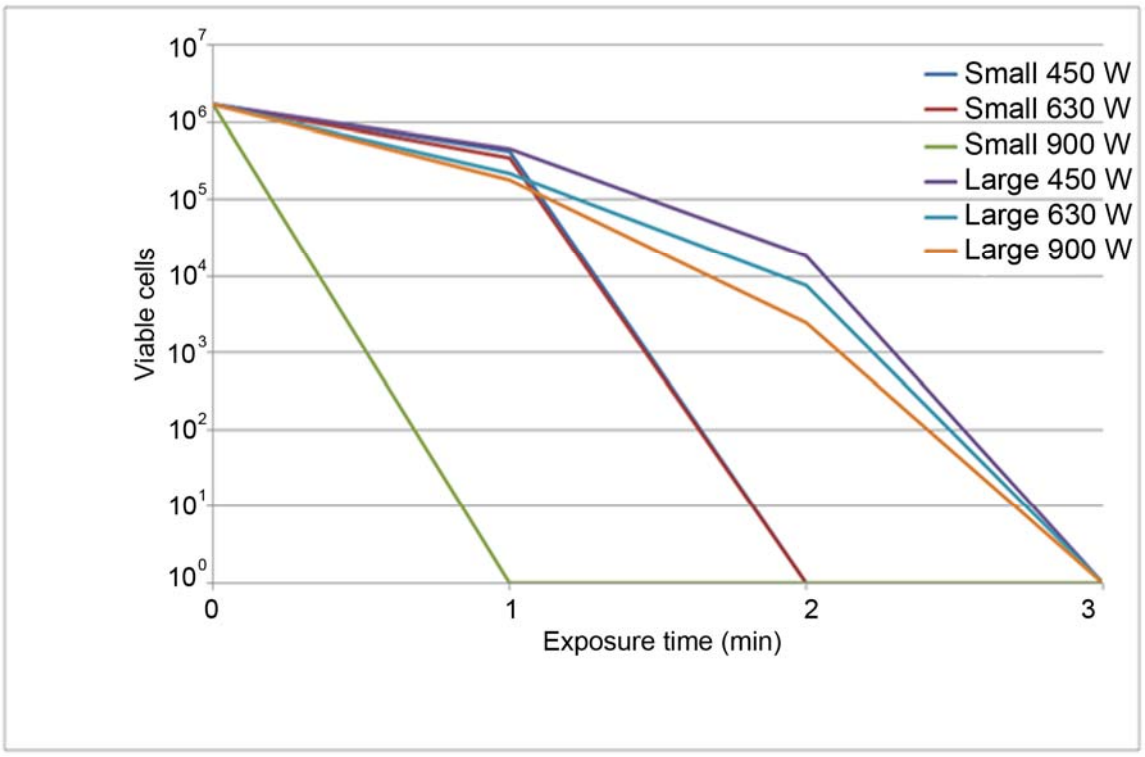

Fig. 1 Remaining viable cells after the different microwave disinfection regimens.

3. Conclusão

Dentro das limitações deste estudo, pode-se concluir que quanto menor a área da prótese removível colonizada por biofilme mais rápido é o processo de desinfecção por meio de irradiação por micro-ondas. A irradiação por micro-ondas durante 3 minutos e baixa potência mostrou-se efetiva para a esterilização das próteses.

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5. Apêndices

Anexo 1: Figuras do capítulo

1. Prótese suporte utilizada para desinfecção por energia de micro-ondas.

2. Matriz metálica para confecção dos discos de resina de PMMA

3. Discos dispostos sobre a prótese suporte e o conjunto imerso em água para a realização do ensaio de desinfecção por energia de micro-ondas.

Anexo 2: Comprovante de submissão

Submission Confirmation

Thank you for submitting your manuscript to *Gerodontology*.

Manuscript ID:	GER-10-OA-0215
Title:	Influence of *Candida albicans* biofilm coverage area on denture disinfection by microwave energy
Authors:	Senna, Plinio Silva, Wander Del Bel Cury, Altair
Date Submitted:	11-May-2010

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COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Desinfecção de próteses dentais removíveis por energia de microondas: Efetividade e efeitos sobre a resina a base de poli(metilmetacrilato)**", protocolo nº **112/2008**, dos pesquisadores **ALTAIR ANTONINHA DEL BEL CURY, PLINIO MENDES SENNA e WANDER JOSÉ DA SILVA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 15/10/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Microwave disinfection of removable dentures: Effectiveness and effects on acrylic resin**", register number **112/2008**, of **ALTAIR ANTONINHA DEL BEL CURY, PLINIO MENDES SENNA and WANDER JOSÉ DA SILVA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 15/10/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

Anexo 4 – Análise dos resultados e testes estatísticos

Obs	disc	time	power	cell	effic	esteril	temp	cell_trans
1	seven	1	350	489523.80	75.52	1	43.7	699.660
2	seven	1	350	457142.90	77.14	1	44.6	676.123
3	seven	1	350	435238.10	78.24	1	44.9	659.726
4	seven	1	350	492381.00	75.38	1	45.0	701.699
5	seven	1	350	398095.20	80.10	1	43.7	630.948
6	seven	1	350	394285.70	80.29	1	44.1	627.922
7	seven	1	500	333333.30	83.33	1	50.6	577.350
8	seven	1	500	327809.50	83.61	1	50.2	572.547
9	seven	1	500	363809.50	81.81	1	50.6	603.166
10	seven	1	500	156190.50	92.19	1	49.6	395.209
11	seven	1	500	228285.70	88.59	1	49.4	477.793
12	seven	1	650	159333.30	92.03	1	50.1	399.166
13	seven	1	650	127047.60	93.65	1	59.1	356.437
14	seven	1	650	151619.10	92.42	1	55.3	389.383
15	seven	1	650	155333.30	92.23	1	58.3	394.123
16	seven	1	650	221142.90	88.94	1	60.9	470.258
17	seven	1	650	206761.90	89.66	1	56.8	454.711
18	seven	1	650	184857.10	90.76	1	59.1	429.950
19	seven	2	350	19438.10	99.03	1	62.1	139.421
20	seven	2	350	18857.14	99.06	1	62.9	137.321
21	seven	2	350	17419.05	99.13	1	64.7	131.981
22	seven	2	350	17411.11	99.13	1	61.3	131.951
23	seven	2	350	16777.78	99.16	1	62.1	129.529
24	seven	2	350	18285.71	99.09	1	61.6	135.225
25	seven	2	500	9973.33	99.50	1	71.2	99.867
26	seven	2	500	6166.67	99.69	1	73.1	78.528
27	seven	2	500	7755.56	99.61	1	71.2	88.066
28	seven	2	500	7320.00	99.63	1	72.2	85.557
29	seven	2	500	5588.89	99.72	1	72.9	74.759
30	seven	2	650	8323.81	99.58	1	72.7	91.235
31	seven	2	650	2500.00	99.88	1	92.3	50.000
32	seven	2	650	2453.33	99.88	1	89.2	49.531
33	seven	2	650	2853.33	99.86	1	92.7	53.417
34	seven	2	650	2780.95	99.86	1	88.1	52.735
35	seven	2	650	1746.67	99.91	1	92.3	41.793
36	seven	2	650	2050.00	99.90	1	94.1	45.277
37	seven	3	350	.	100.00	2	78.1	.
38	seven	3	350	.	100.00	2	77.0	.
39	seven	3	350	.	100.00	2	75.5	.
40	seven	3	350	.	100.00	2	73.9	.
41	seven	3	350	.	100.00	2	78.1	.
42	seven	3	350	.	100.00	2	79.2	.
43	seven	3	500	.	100.00	2	89.0	.
44	seven	3	500	.	100.00	2	90.5	.
45	seven	3	500	.	100.00	2	93.9	.
46	seven	3	500	.	100.00	2	89.0	.
47	seven	3	500	.	100.00	2	91.5	.

48	seven	3	650	.	100.00	2	90.5	.
49	seven	3	650	.	100.00	2	98.6	.
50	seven	3	650	.	100.00	2	98.0	.
51	seven	3	650	.	100.00	2	92.8	.
52	seven	3	650	.	100.00	2	99.1	.
53	seven	3	650	.	100.00	2	96.2	.
54	seven	3	650	.	100.00	2	98.3	.
55	three	1	350	502222.2	74.89	1	43.7	708.676
56	three	1	350	253333.3	87.33	1	44.6	503.322
57	three	1	350	577777.8	71.11	1	44.9	760.117
58	three	1	350	337777.8	83.11	1	45.0	581.187
59	three	1	350	548888.9	72.56	1	43.7	740.870
60	three	1	350	260000.0	87.00	1	44.1	509.902
61	three	1	500	428888.9	78.56	1	50.6	654.896
62	three	1	500	313333.3	84.33	1	50.2	559.762
63	three	1	500	324444.4	83.78	1	50.6	569.600
64	three	1	500	273333.3	86.33	1	49.6	522.813
65	three	1	500	322222.2	83.89	1	49.4	567.646
66	three	1	650	368888.9	81.56	1	50.1	607.362
67	three	1	650	.	100.00	2	59.1	.
68	three	1	650	.	100.00	2	55.3	.
69	three	1	650	.	100.00	2	58.3	.
70	three	1	650	.	100.00	2	60.9	.
71	three	1	650	.	100.00	2	56.8	.
72	three	1	650	.	100.00	2	59.1	.
73	three	2	350	.	100.00	2	62.1	.
74	three	2	350	.	100.00	2	62.9	.
75	three	2	350	.	100.00	2	64.7	.
76	three	2	350	.	100.00	2	61.3	.
77	three	2	350	.	100.00	2	62.1	.
78	three	2	350	.	100.00	2	61.6	.
79	three	2	500	.	100.00	2	71.2	.
80	three	2	500	.	100.00	2	73.1	.
81	three	2	500	.	100.00	2	71.2	.
82	three	2	500	.	100.00	2	72.2	.
83	three	2	500	.	100.00	2	72.9	.
84	three	2	650	.	100.00	2	72.7	.
85	three	2	650	.	100.00	2	92.3	.
86	three	2	650	.	100.00	2	89.2	.
87	three	2	650	.	100.00	2	92.7	.
88	three	2	650	.	100.00	2	88.1	.
89	three	2	650	.	100.00	2	92.3	.
90	three	2	650	.	100.00	2	94.1	.
91	three	3	350	.	100.00	2	78.1	.
92	three	3	350	.	100.00	2	77.0	.
93	three	3	350	.	100.00	2	75.5	.
94	three	3	350	.	100.00	2	73.9	.
95	three	3	350	.	100.00	2	78.1	.
96	three	3	350	.	100.00	2	79.2	.
97	three	3	500	.	100.00	2	89.0	.
98	three	3	500	.	100.00	2	90.5	.
99	three	3	500	.	100.00	2	93.9	.
100	three	3	500	.	100.00	2	89.0	.

Obs	disc	time	power	cell	effic	esteril	temp	cell_trans
101	three	3	500	.	100	2	91.5	.
102	three	3	650	.	100	2	90.5	.
103	three	3	650	.	100	2	98.6	.
104	three	3	650	.	100	2	98.0	.
105	three	3	650	.	100	2	92.8	.
106	three	3	650	.	100	2	99.1	.
107	three	3	650	.	100	2	96.2	.
108	three	3	650	.	100	2	98.3	.

The ANOVA Procedure

Class Level Information

Class	Levels	Values
disc	2	seven three

Number of observations 108

Dependent Variable: esteril

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	5.33333333	5.33333333	26.50	<.0001
Error	106	21.33333333	0.20125786		
Corrected Total	107	26.66666667			

R-Square	Coeff Var	Root MSE	esteril Mean
0.200000	28.83971	0.448618	1.555556

Source	DF	Anova SS	Mean Square	F Value	Pr > F
disc	1	5.33333333	5.33333333	26.50	<.0001

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The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for esteril

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	106
Error Mean Square	0.201258
Critical Value of Studentized Range	2.80382
Minimum Significant Difference	0.1712

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	disc
A	1.77778	54	three
B	1.33333	54	seven

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The GLM Procedure

Class Level Information

Class	Levels	Values
time	3	1 2 3
power	3	350 500 650

Number of observations 108

The GLM Procedure

Dependent Variable: esteril

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	14.23809524	1.77976190	14.18	<.0001
Error	99	12.42857143	0.12554113		
Corrected Total	107	26.66666667			

R-Square	Coeff Var	Root MSE	esteril Mean
0.533929	22.77757	0.354318	1.555556

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	2	13.09746328	6.54873164	52.16	<.0001
power	2	0.52380952	0.26190476	2.09	0.1296
time*power	4	1.04761905	0.26190476	2.09	0.0883

The GLM Procedure

Tukey's Studentized Range (HSD) Test for esteril

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	0.125541
Critical Value of Studentized Range	3.36511
Minimum Significant Difference	0.1987

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	time
A	2.00000	36	3
B	1.50000	36	2
C	1.16667	36	1

The GLM Procedure

Tukey's Studentized Range (HSD) Test for esteril

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	0.125541
Critical Value of Studentized Range	3.36511

Comparisons significant at the 0.05 level are indicated by ***.

power Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
650 - 500	0.14286	-0.05868	0.34440
650 - 350	0.14286	-0.04863	0.33435
500 - 650	-0.14286	-0.34440	0.05868
500 - 350	0.00000	-0.20842	0.20842
350 - 650	-0.14286	-0.33435	0.04863
350 - 500	0.00000	-0.20842	0.20842

The CORR Procedure

2 Variables: esteril temp

Simple Statistics

Variable	N	Mean	Std Dev	Median	Minimum	Maximum
esteril	108	1.55556	0.49922	2.00000	1.00000	2.00000
temp	108	71.51667	18.13286	72.45000	43.70000	99.10000

Pearson Correlation Coefficients, N = 108
Prob > |r| under H0: Rho=0

	esteril	temp
esteril	1.00000	0.61702 <.0001
temp	0.61702 <.0001	1.00000