UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO

MESTRADO EM ODONTOLOGIA

ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA-MATERIAIS DENTÁRIOS

INFLUÊNCIA DA ADIÇÃO DE MICROESFERAS CONTENDO AMOXICILINA NAS PROPRIEDADES FÍSICAS, QUÍMICAS E BIOLÓGICAS DE UM CIMENTO ENDODÔNTICO EXPERIMENTAL

Nélio Bairros Dornelles Junior

Vicente Castelo Branco Leitune

Orientador

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Mahatma Gandhi

NOTA PRELIMINAR

A presente dissertação foi redigida segundo a Resolução nº 093/2007 do Conselho de Ensino, Pesquisa e Extensão da Universidade Federal do Rio Grande do Sul. Enquadra-se na forma descrita no item “b” do artigo 3º da resolução: ”Tese, Dissertação ou Trabalho de Conclusão de Curso que contenham artigo(s) prontos para submissão a publicação;”

O manuscrito será enviado para publicação na Revista *International Endodontic Journal.*

Resumo

O objetivo do estudo foi desenvolver um cimento endodôntico com microesferas contendo amoxicilina e avaliar suas propriedades. As microesferas foram produzidas por secagem e caracterizadas por Microscopia eletrônica de varredura (MEV) e difração a laser. A formulação da resina base para um cimento endodôntico dual foi obtida pela mistura, em massa, de 70% de UDMA, 15% de GDMA e 15% de BISEMA. Como sistema iniciador/ativador, foram incorporados canforoquinona, DHEPT e peróxido de benzoíla, a 1% em mol e BHT em 0,01% em massa. Foram adicionados à resina base, em massa, 10 e 15% de microesferas de amoxicilina, além de um grupo sem microesferas (controle). Em todos os grupos foi adicionado trifluoreto de itérbio (10% em massa) como agente radiopacificante. Os cimentos foram avaliados quanto ao grau de conversão (GC) por Raman (n=3) imediatamente e após 24 horas de armazenamento, degradação em solvente (n=3) após 1 hora de imersão em álcool 70%, perfil de liberação do fármaco (n=3), atividade antimicrobiana contra *Enterococcus Faecallis* (n= 3), escoamento (n=3) e espessura de película (n=3)e citotoxicidade (n=3). Os dados foram analisados por ANOVA e Tukey com nível de significância de 5%. As microesferas apresentaram diâmetro médio de 2,664 µm. O grau de conversão imediato variou entre 51,73% e 55,13% e em 24h variou entre 60,79% e 73,80% sem apresentar diferença estatística entre os grupos. O percentual de degradação em solvente apresentou diferença significativa entre o grupo controle e o grupo com 15% de microesferas, variando entre 54,44% e 56,21% de redução. O perfil de liberação do fármaco mostrou que em 96h ocorreu uma liberação média de 73,76% do fármaco. A atividade antimicrobiana apresentou redução

significativa dos grupos experimentais em 24 e 48h. Em 96h o grupo com 15% não apresentou diferença estatística quando comparado ao grupo controle ($p>0,05$). O escoamento apresentou uma redução significativa nos grupos experimentais comparados ao grupo controle ($p<0,05$). A espessura de película variou, mas não apresentou diferença estatística entre os grupos ($p=0,63$). A citotoxicidade apresentou alta viabilidade celular no tempos avaliados. Com base nesses resultados, pode-se concluir que a adição de até 10% de microesferas contendo amoxicilina apresentou característica antimicrobiana e não alterou as propriedades do cimento endodôntico experimental.

Palavras-chave

Amoxicilina. Cimentos endodônticos, Metacrilatos. Microesferas.

Abstract

The objective of the study was to develop an endodontic sealer with amoxicillin-loaded microsphere and to evaluate its properties. The microspheres were produced by drying and characterized by Scanning Electron Microscopy (SEM) and laser diffraction. The formulation of the base resin dual cure endodontic cement was obtained by mixing, by weight, 70% UDMA, 15% GDMA and 15% BISEMA. As initiator / activator system, camphorquinone, DHEPT and benzoyl peroxide, 1 mol% and BHT were incorporated in 0.01% by weight. 10 and 15% of amoxicillin microspheres were added to the base resin, in addition to a group without microspheres (control). Ytterbium trifluoride (10% by weight) as radiopacifier was added in all groups. The cements were evaluated for Raman (n=3) conversion grade immediately after 24 hours storage, solvent degradation (n=3) after 1 hour immersion in 70% alcohol, drug release profile (n=3), antimicrobial activity against E. faecallis (n=3), flow (n=3) and film thickness (n=3) and cytotoxicity (n=3). Data were analyzed by ANOVA and Tukey with significance level of 5%. The microspheres had an average diameter of 2,664 μm. The degree of immediate conversion ranged from 51.73% to 55.13%, and in 24h conversion ranged from 60.79% to 73.80%, with no statistical difference between the groups. The percentage of degradation in solvent showed a significant difference between the control group and the group with 15% of microspheres, varying between 54.44% and 56.21% reduction. The drug release profile showed that a mean release of 73.76% of the drug occurred in 96h. The antimicrobial activity showed a significant reduction of the experimental groups in 24 and 48h. In 96h the group with 15% presented no statistical difference when compared to the control group ($p > 0.05$). The flow

showed a significant reduction in the experimental groups compared to the control group ($p < 0.05$). The film thickness varied, but did not present statistical difference between the groups ($p = 0.63$). Cytotoxicity showed high cellular viability at the determined times. Based on these results, it can be concluded that the addition of up to 10% of microspheres containing amoxicillin showed antimicrobial characteristics and did not alter the properties of the experimental endodontic cement.

Keywords

Amoxicillin. Endodontic sealers, Methacrylates. Microspheres.

Sumário

1. Introdução

O objetivo do tratamento endodôntico é a eliminação ou redução dos microrganismos do sistema de canais radiculares (Baer e Maki, 2010) e prevenir a reinfecção subsequente (Pizzo *et al.*, 2006). Isto é conseguido por meio de limpeza mecânica, irrigação e medicação (Waltimo *et al.*, 2001; Sousa *et al.*, 2010; Nirupama *et al.*, 2014; Vemisetty *et al.*, 2014), seguida da obturação tridimensional do espaço do canal radicular (Pizzo *et al.*, 2006).

O ambiente endodôntico proporciona um meio favorável para o estabelecimento de uma microbiota predominantemente anaeróbia (Nair, 2004; Skucaite *et al.*, 2009), sendo anaeróbios gram-positivos e facultativos as espécies mais frequentemente encontradas dentro de condutos radiculares tratados e ao redor da área periapical (Zhang *et al.*, 2015).

O biofilme endodôntico possui uma grande diversidade de microrganismos, entre eles *Enterococcus Faecalis* que possuem uma alta capacidade de formar biofilmes sob diferentes condições ambientais e nutricionais (Jhajharia *et al.*, 2015) sendo resistente a muitos agentes antimicrobianos (Kayaoglu e Orstavik, 2004). Os *Enterococcus* apresenta formato ovóide, são gram-positivos, anaeróbios facultativos, podendo ocorrer isoladamente, em pares ou em cadeias curtas que são alongadas em forma de corrente. Não possuem capacidade de formas endosporos e podem ser móveis (Schleifer e Kilper-Balz, 1984). O *Enterococcus Faecalis* apresenta também metabolismo fermentativo (Zhang *et al.*, 2015).

A prevalência de *E. faecalis* está associada a diferentes formas de doença periapical, sendo primaria ou secundária/persistente. A infecção

primária é entendida como uma infecção aguda (sintomática) causada, por exemplo, um caso de necrose pulpar. A secundária é causada por microrganismos que permanecem no sistema de canal radicular depois do tratamento endodôntico de um caso primário e pode levar à formação de periodontite apical assintomática (Skucaite *et al.*, 2009). Nos casos de doença periapical primária sua prevalência varia de 4 a 40%, na persistente varia entre 24 a 77% (Rocas *et al.*, 2004). Um estudo avaliou a prevalência de *E. faecallis* em tratamento endodôntico que necessitava de retratamento em virtude da presença de lesão periapical e mostrou uma prevalência de 38% de *E. Faecalis* (Wang *et al.*, 2012).

O controle da infecção não depende apenas da remoção de bactérias do sistema de canais, mas também de se evitar a reinfecção através de uma boa obturação (Grossman, 1980) tridimensional dos condutos (Schilder, 2006). A utilização de cimentos que contenham alguma atividade antimicrobiana é considerada benéfica para reduzir o número de microrganismos remanescentes e erradicar a infecção (Shih *et al.*, 2014), pois é difícil eliminar o *Enterococcus faecalis* dos túbulos dentinários com o uso de medicamentos intracanal (Sharma *et al.*, 2014)

O cimento endodôntico ideal deve ter estabilidade dimensional, apresentar boa atividade antimicrobiana e baixa toxicidade aos tecidos periapicais (Grossman, 1980). Além disso, deve ser capaz de se ligar à dentina do canal radicular e a guta-percha, evitando infiltração. Com os avanços na tecnologia adesiva, a introdução de uma nova geração de cimentos que se baseiam em propriedades adesivas (Pameijer e Zmener, 2010), com dimetracrilatos aromáticos parecem ser uma alternativa útil (Ogliari *et al.*, 2008).

Buscando-se agregar outras propriedades aos cimentos de uso endodôntico, cargas inorgânicas bioativas são incorporadas a cimentos experimentais, tais como fosfatos de cálcio (Portella *et al.*, 2015). Já os efeitos antimicrobianos são controversos nos cimentos comerciais à base de resina epóxica (Pizzo *et al.*, 2006; Slutzky-Goldberg *et al.*, 2008; Faria-Junior *et al.*, 2013). Por outro lado, cimentos a base de eugenol, de hidróxido de cálcio e biocerâmicos, mostraram limitada atividade antimicrobiana (Cobankara *et al.*, 2004; Pizzo *et al.*, 2006; Slutzky-Goldberg *et al.*, 2008; Faria-Junior *et al.*, 2013).

A atividade antimicrobiana tem sido estudada também em cimentos endodônticos, uma vez que bactérias viáveis permanecem nos túbulos dentinários e canais laterais mesmo após o preparo químico-mecânico (Lin *et al.*, 1992). Assim antibióticos e outros agentes já foram incorporados a cimentos endodônticos, melhorando as propriedades antimicrobianas destes materiais (Holt *et al.*, 2007; Baer e Maki, 2010; Bidar *et al.*, 2012; Gjorgievska *et al.*, 2013), e mantendo propriedades como espessura de película e solubilidade dentro dos padrões estabelecidos pela ISO 6876:2012 (Razmi *et al.*, 2010). O sucesso da terapia endodôntica pode ser melhorado com o uso de cimentos que apresentam excelente capacidade de vedação e propriedades antimicrobianas. Isto permite que o cimento possa reagir melhor com infecção e prevenir que bactérias penetrem novamente no conduto (Aravind. *et al.*, 2006).

A administração de antibióticos sistêmicos muitas vezes tem sido utilizada como complemento ao tratamento endodôntico sendo indicada em casos de comprometimento sistêmico incluindo febre, linfodenopatia, risco de bacteremia (Skucaite *et al.*, 2009), pois depende da colaboração do paciente e

da absorção do fármaco no trato gastrointestinal (Mohammadi e Abbott, 2009). Embora a grande maioria das infecções de origem endodôntica sejam tratadas sem a necessidade de antibióticos, esses fármacos são utilizadas na prática clínica (Skucaite *et al.*, 2009).

A amoxicilina é um antibiótico bactericida, de amplo espectro, beta-lactâmico que inibe a síntese da parede celular bacteriana (Majumdar e Pratt, 2009). As penicilinas são os agentes antimicrobianos mais utilizados devido à sua eficácia histórica, baixa toxicidade e baixo custo para infecções odontogênicas (Pinheiro *et al.*, 2004). Após a administração sistêmica de um antibiótico, é improvável que a concentração que atinge o canal radicular iniba o crescimento bacteriano (Saber Sel e El-Hady, 2012). A principal vantagem dos antibióticos locais em comparação com o uso sistêmico é que as complicações sistêmicas são evitadas e concentrações mais altas atingem as áreas-alvo (Sharma *et al.*, 2014). Dentes endodonticamente tratados e lesões perirradiculares crônicas associadas à necrose pulpar não possuem suprimento sanguíneo adequado, portanto a concentração de antibióticos administrados sistemicamente não atinge o sistema de canais radiculares, não trazendo benefícios ao tratamento (Molander *et al.*, 1998).

Novas abordagens envolvem o uso de fármaco local por meio de sistemas de entrega baseados em partículas de diferentes tamanhos: micropartículas / nanopartículas feita a partir de polímeros biocompatíveis. Tais dispositivos permitem a introdução de agentes antimicrobianos ou outros fármacos diretamente na bolsa periodontal, ou dentro do canal radicular, e a libertação prolongada de concentrações constantes de estes agentes contribui para um melhor controle de infecções (Puri e Puri, 2013). Aliado ao uso tópico

de fármacos, a encapsulação desses agentes pode ser uma alternativa para incorporação em materiais de uso odontológico na busca de ação terapêutica por um período prolongado mantendo propriedades mecânicas. Em geral, os sistemas carreadores de substância ativa podem ter dimensões micro ou nanométricas, sendo esferas, constituídas basicamente por polímero, ou cápsulas, compostas por uma parede polimérica ao redor de um núcleo oleoso que contém o fármaco (Mora-Huertas *et al.*, 2010).

A liberação controlada por um período prolongado é altamente benéfica para fármacos que são rapidamente metabolizados e eliminados do corpo após sua administração. Este sistema de encapsulação apresenta como principal vantagem melhorar estabilidade do fármaco (Ourique *et al.*, 2008), biocompatilibilidade com os tecidos, pois utiliza polímeros biocompatíveis e biodegradáveis (GuinebretièRe *et al.*, 2002) e também apresenta liberação controlada (Hernandez *et al.*, 2013). Os fármacos encapsuladas são liberados por difusão, degradação ou bioerosão do invólucro polimérico (Musyanovych e Landfester, 2014).

2. Objetivo

O objetivo deste trabalho foi desenvolver um cimento endodôntico de dupla ativação contendo microesferas de amoxicilina e avaliar suas propriedades.

3. Manuscrito

Influence of the addition of amoxicillin-loaded microsphere in an experimental endodontic sealer

N B Dornelles Junior[1]
F M Collares[1]
B Genari[1]
S M W Samuel[1]
R A Arthur[2]
F Visioli[3]
S S Guterres[4]
V C B Leitune[1]

[1]Laboratory of Dental Materials, [2]Laboratory of Microbiology, [3] Laboratory of Pathology School of dentistry, [4]Laboratory of Micro and Nanoparticles Applied in Therapeutics of School of Pharmacy, Federal University of Rio Grande do Sul.

Running title: Endodontic sealer with microsphere load amoxicillin
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*corresponding Author
Vicente Castelo Branco Leitune
Laboratory of Dental Materials, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos 2492, Porto Alegre, RS, Brazil, 90035-003.
Telephone: 33085198
E-mail: vicente.leitune@ufrgs.br

Abstract

Aim: To develop an endodontic sealer with amoxicillin-loaded microsphere and to evaluate its properties.

Methods: The microspheres were produced by drying in spray dryer. The sealer was obtained by mixing 70 wt% UDMA, 15 wt% GDMA and 15 wt% BISEMA. In addition, CQ, DHEPT and PB were incorporated at 1 mol% and 0.01 wt% BHT and 10 wt% YbF3. Two experimental groups were formulated (10 and 15 wt% of microspheres) and one control group, without microspheres. The sealers were evaluated for the degree of conversion (n=3), degradation in solvent (n=3), drug release profile (n=3), antimicrobial activity (n=3), flow (n=3), film thickness (n=3) and cytotoxicity (n=3). Data were analyzed by ANOVA and Tukey with significance level of 5%.

Results: Microespheres presented a mean size of 2.664 µm. The immediate degree of conversion ranged from 51.73% to 55.13% and the 24h degree of conversion ranged from 60.79% to 73.80%. The degradation in solvent ranged between 54.44% and 56.21% reduction. The drug release profile showed an average release of 73.76% of the drug in 96h. The antimicrobial activity showed a significant reduction of the experimental groups in 24 and 48h. The flow and film thickness showed values in accordance to the ISO 6876. Cytotoxicity showed high cellular viability.

Conclusion: The addition of up to 10% of microspheres containing amoxicillin presented antimicrobial activity and did not alter the properties of the experimental endodontic cement.

Introduction

Remaining bacteria could be viable within the root canal system after mechanical preparation (Lin et al. 1992). The presence of a resistant microorganism is the major cause of endodontic failure (Hoelscher et al. 2006; Gong et al. 2014; Nirupama et al. 2014). Anaerobic gram-positive and facultative microorganism are the most commonly found in treated root canal and in the periapical area (Zhang et al. 2015). *Enterococcus faecalis* have the ability to penetrate dentinal tubules on different environmental and nutritional conditions (Jhajharia et al. 2015). Furthermore the presence of this microorganism is associated to acute infections after root canal treatment (Skucaite et al. 2009).

The control of the infection depends not only on bacteria removal, but also to avoid reinfection through a completely sealing the canal system (Pizzo et al. 2006; Schilder 2006). The use of root canal filling material with antimicrobial activity is considered beneficial to reduce the number of remaining microorganisms (Shih et al. 2014). Few studies have been performed to evaluate the incorporation of antibiotics to endodontic sealers with improved antimicrobial property (Razmi et al. 2008). Commercial amoxicillin was added to cements showed a significant difference in antimicrobial response against *E. faecalis* (Hoelscher et al. 2006). Nevertheless, no encapsulated drug was added to endodontic cements.

New approaches involve the use of local drug-carrier system based on micro or nano particles to allow the incorporation of antimicrobials with prolonged release (Puri & Puri 2013). This system improves the stability of the

drug (Ourique et al. 2008) biocompatibility (Guinebretière et al. 2002) and the control of drug delivery (Hernandez et al. 2013). Local carrier system have been used in endodontics for cleaning channels with calcium hydroxide (Strom et al. 2012). However, the use of this system has been few explored in dentistry (Baer & Maki 2010).

The aim of this study was to develop a microsphere containing amoxicillin (Amx-ME), incorporate into an experimental sealer and evaluate the properties.

Materials and Methods

This study was an in vitro study approved by the local ethics committee (nº 1.739.340).

Preparation of experimental endodontic sealer

The experimental endodontic sealer was obtained by mixing 70 wt% urethane dimethacrylate (UDMA), 15 wt% ethoxylated bisphenol A glycol dimethacrylate (BISEMA30) and 15 wt% glycerol 1,3-dimethacrylate (GDMA). Camphorquinone (CQ), N,N-dihydroxyethyl-para-toluidine (DHEPT) and benzoyl peroxide (BP) were added as photoinitiator / activator system to produce a dual-cure resin-based experimental sealer. It was also added 10 wt% ytterbium trifluoride (YbF_3) as radiopacifier, and 0.01 wt% Butylated hydroxytoluene (BHT) as polymerization inhibitor.

Preparation of microsphere

Amoxicillin-loaded microspheres (Amx-ME) were prepared with Eudragit® S100, poly(MMA-co-MAA) (3.0 g) and trihydrate amoxicillin (0.3 g). The polymer was first dissolved in acetone (200 mL) and subsequently the amoxicillin was added under magnetic stirring at 25 °C. The suspension was spray dried (B-290, Buchi, Flawi, Switzerland) in the closed cycle dryer, using nitrogen as an inert gas. The inlet temperature in the drying chamber was maintained at approximately 60 ± 4°C, and the outlet temperature was 40 ± 4°C.

Drug release

Three discs with 0.2 g of experimental sealer containing 15 % of Amx-ME were immersed in volumetric flasks with 10 mL of simulated body fluid (SBF) under magnetic stirring at 37 °C. After 24, 48 e 96h, 1 mL of released medium was collected and fresh SBF was replaced. The aliquots were filtered using a 0.45-µm (Millipore) filter. Free amoxicillin was measured using high-performance liquid chromatography (HPLC, Shimadzu LC 10-A Shimadzu, Kyoto, Japan) with injector S-200, a UV/visible detector (λ = 280 nm), a guard column, and Nova-Pak® C18 3.9 × 150 mm (4 µm) Waters column. The mobile phase (60/40 v/v acetonitrile/water solution, pH 4.5, adjusted with acetic acid) was filtered and pumped at a constant flow rate of 1 mL min^{-1}. After injection of 20 µL, amoxicillin was detected with a retention time of 1.9 seconds.

Antimicrobial test

Antimicrobial test was based previous study (Altmann et al. 2015). For antibacterial activity evaluation of experimental sealer, three specimens (disks of 3 mm diameter x 2 mm thickness) of each group were fixed on teflon matrixes on the lid of a 48-well plate and sterilized by ethylene oxide. In the sterile 48-well plate, 900µL of brain heart infusion (BHI) broth (Sigma-Aldrich, St Louis, MO, USA) with 90µL of a suspension of an overnight broth culture of *Enterococcus faecallis* (ATCC 29212), adjusted to optical density of 0.3 (550nm) were added to each one of the wells. The plate was closed and incubated at 37°C for 24, 48, and 96 hours. The samples from each group were then removed from the lid's teflon matrixes and placed inside a micro-tube containing 900µL of saline and vortexed. Dilutions were made up to 10^{-6}. Two 25µL-drops of each dilution were platted in BHI agar Petri dishes and incubated for 48 hours at 37°C. The number of colony forming units (CFU) was visually counted by optical microscopy and transformed to log CFU/mL.

Cytotoxicity sulforhodamine B (SRB) colorimetric assay

Obtaining dental pulp fibroblasts

Fibroblasts were derived from two intact human third molars with incomplete root formation of different patients without systemic health problems. Patients were asked to donate your tooth for the study and agreed and signed the Informed Consent and Informed (Sipaviciute & Maneliene). After the

extractions, the teeth were immersed in 1 mL of Dulbecco's Modified Eagle Medium (DMEM), supplemented with Hepes, fetal bovine serum 10% and 100 IU/mL penicillin, 100 µg/mL streptomycin (Thermo Fischer Scientific, Waltham, Massachusets, EUA), at room temperature, to transport to the laminar flow hood. The pulp was removed using endodontic instruments and dentin excavator. The collected tissue was sectioned into small fragments with scalpel blade. Tissue fragments were placed in cell culture plates in DMEM with Hepes, fetal bovine serum 10% and 100 IU/mL penicillin, 100 µg/mL streptomycin (Thermo Fischer Scientific, Waltham, Massachusets, EUA), and then incubated at 37˚C humidified atmosphere with 5% CO_2. Culture medium was changed 24 hours after the initial plating and from this moment on, every 2 days. After expansion to reach sufficient numbers of cells, experiments were performed with cells in fifth passage.

SRB Cytotoxicity Assay

Fibroblasts obtained from human dental pulp were used for the cytotoxicity assay. Eluate was prepared immersing the specimen (3mm diameter x 1mm thickness) in 1mL of medium during 24 hours and 72 hours. Cells were seeded in triplicate at a concentration of 5 x 10^3 in 96 well plates, and then treated with 100µL of eluate. After 72 hours cells were fixed with a concentration of 10% trichloroacetic acid (TCA), they were incubated at 4° C for 1 hour and subsequently were washed 6 times under running water and dried at room temperature. Sulforhodamide B (SRB, Sigma-Aldrich, St. Louis, USA) at

4% was added to stain the cells and the plate was incubated for 30 minutes at room temperature. The plate was washed 4 times with 1% acetic acid to remove unbound excess dye and allowed to dry completely at room temperature. Trizma solution was added and the plate incubated for 1 hour to allow complete solubilization of the dye. At the end of this process the microplates were read at 560nm. Cell viability was normalized against initial control and control free of drugs and expressed in percentage.

Flow test

The flow test was conducted according to ISO 6876 (Standardization 2001). A total of 0.05 mL of each experimental sealer was placed on a glass plate (40 x 40 x 5 mm) with a graduated syringe. At 180 ± 5 s after mixing was started, another plate with a mass of 20 ± 2 g and a load of 100 g was placed on top of the material. Ten minutes after mixing had been started, the load was removed, and the major and minor diameters of the compressed material were measured using a digital caliper. If both measurements were within 1 mm of each other, the results were recorded. If the major and minor diameter discs were not uniformly circular or did not fall within 1 mm of each other, the test was repeated. The test was conducted three times for each experimental group, and the mean value was recorded.

Film Thickness

The film thickness was evaluated according to ISO 6876 (Standardization 2001). Two glass plates with an area of contact area of 200 ± 10 mm^2 each will have their thicknesses measured added. The centre of one of the plates was covered with 0.05 mL of experimental sealer, and a second plate was placed on top of the material. At 180 ± 5 s after the start of mixing, a load of 150 N was applied vertically on top of the glass plate. Ten minutes after the mixing had been started, the thickness of the two glass plates and the interposed sealer film was measured using a digital caliper. The difference in the thickness of the two glass plates, with and without sealer, was recorded as the film thickness of the experimental sealer material. The mean value of three measurements for each sealer was recorded as the film thickness of the material.

Softening in solvent

To evaluate the softening in solvent was used the same samples of degree of conversion. These specimens were embedded in acrylic resin and subjected to polishing on an electric polisher (Model 3V, AROTEC, Cotia, SP, Brazil) using water sanding with granulation 600.1200, 2000 and felt disc with polishing paste aluminum oxide suspension – 0.05µm (Fortel, São Paulo, SP, Brazil). The Knoop microhardness were evaluated using a digital microhardness tester (HMV-2, Shimadzu, Tokyo, Japan) with a load of 10g for a time of 5 s. Three indentations were held on the surface of each specimen and hardness will be given by the average of the measurements. The initial hardness was

measured (KHN1), and after immersion in 70% alcohol (70% absolute ethanol and 30% distilled water) for 1 h, the final hardness was obtained (KHN2). The reduction of hardness, as a percentage, was calculated by the equation 1:

$$\Delta KHN\% = \frac{KHN1 - KHN2}{KHN1} \quad (1)$$

Degree of conversion

The degree of conversion (DC) was evaluated by Raman spectroscopy (SENTERRA Bruker Optics, Ettlingen, Germany). Five samples were used for each group. For each sample of 0.03 g of cement was mixed on and placed in a polyvinylsiloxane matrix of 4 mm diameter and 1 mm in height. The degree of conversion was measured at 3 points with exposure parameters 3s and 5 co-additions using a diode laser of 785 nm with a resolution of 3-5 cm^{-1}. The samples were light cured for 40 seconds (RadiiCal, SDI, Bayswater, Vic., Austrália). After 24 hours, the same samples were analyzed again. To calculate the intensity (peak height) of vibration of the aliphatic carbon double bonds in 1640 cm^{-1} and aromatic carbon-carbon bonds in 1610 cm^{-1} samples polymerized and unpolymerized. DC was determined by the following equation 2:

$$DC = 1 - \left(\frac{\text{absorbance 1640 } cm^{-1}/(\text{absorbance 1610 } cm^{-1} \text{ polymer})}{\text{absorbance 1640 } cm^{-1}/(\text{absorbance 1610 } cm^{-1} \text{ monomer})} \right) \times 100 \quad (2)$$

Statistical analysis

Statistical analysis was performed using one-way ANOVA for flow, film thickness, ethanol softening, antimicrobial test and cytotoxicity. Anova two-way repeated measures was used for the degree of conversion. The paired t-test was used for comparison between the initial and final Knoop microhardness.

All tests were performed with $\alpha = 0.05$.

Results

Microspheres presented a mean size of 2.664 µm. During the first 96 hours of evaluation of 73.76% the drug was released (figure 1). The antimicrobial activity (Figure 1) showed a statistically significant decrease ($p<0.05$) from experimental groups to control group at 48 hours. At 96h only the group with 10% Amx-ME showed a decrease compared to the control group ($p=0.04$). Figure 2 shows the cytotoxic effect of experimental sealer. Cell viability of the experimental groups remained high in both 24 and 48 hours. The flow, film thickness, softening in solvent and degree of conversion values are shown in Table 1. The flow significantly decreased in the experimental groups compared to the control group ($p <0.05$). The film thickness values showed no statistical difference between groups ($p = 0.63$). The microhardness decreased significantly between the initial and final measurement after softening in solvent in all groups ($p<0.05$), However, the group 15% Amx-ME showed a greater degradation percentual after softening in solvent compared of group control

($p<0.001$). The degree of conversion increased from immediate to 24 hours for all groups with no statistical difference between the groups ($p=0.94$).

Discussion

Endodontically treated teeth and chronic periradicular lesions associated with pulpal necrosis do not have adequate blood supply, therefore the concentration of antibiotics administered systemically in the root system is not beneficial (Molander et al. 1998). The present study introduces a novel approach of controlled drug delivery to an endodontic sealer. Microspheres containing amoxicillin were successfully produced and incorporated to an experimental endodontic sealer, presenting drug controlled release with consequent antimicrobial effect. The addition of 10% of amoxicillin microspheres did not compromise its physicochemical properties.

Nanocapsules are a possibility of drug encapsulation for slow release. The drugs must possess properties that allow the encapsulation. The *log* D value is a physico-chemical parameter in terms of predicting the possibility to encapsulate drug and its distribution in nanocapsules. This coefficient is based in the lipophilicity of drugs estimated by logarithm of octanol-water distribution (Oliveira 2013). Independent of the buffer composition of the aqueous phase in the systems, the *log* D value for amoxicillin is -3.4 ± 0.13, indicating a limited possibility to encapsulate the drug (Oliveira 2013; Ferreira 2015). Microspheres can be considered as particles to carrier amoxicillin due to its water solubility, which occurs through β-lactam radical cleavage (Tsuji et al. 1978). The

employed methodology resulted in dried microspheres, whose drug was adsorbed in polymer. Due to distribution of drug in the carrier system, release occurs through diffusion of amoxicillin through polymer (de la Torre et al. 2003); (Mora-Huertas et al. 2010).

One of the consequences of drug release, considering the fact that *E. faecalis* is persistent and the major responsible by failure of endodontic treatment (Rocas et al. 2004; Hoelscher et al. 2006; Wang et al. 2012; Gong et al. 2014; Nirupama et al. 2014) is antimicrobial effect prolonged. In the present study, sealer containing 10% of amoxicillin-loaded microspheres presented antimicrobial effect against *E. Faecalis*. Similar results were shown in some studies that used non-encapsulated commercial amoxicillin (Hoelscher et al. 2006; Sharma et al. 2014). However, carrier systems with controlled drug release have been resulted in lower drug resistance by organism (Peer 2007).

Chemical affinity between cement and microspheres guaranteed by the use of methacrylate-based polymers favors the maintenance of degree of conversion, as shown in previous study (Genari et al. 2016). Eudragit® S100 is an anionic copolymer of methacrylate and the literature has reported its use for late drug delivery (Nandy & Mazumder 2014). Drug distribution and its release through diffusion among chains into polymer could influence the physical-chemical properties of cement. In the root canal, the sealer can be exposed to tissue fluid and exudate and is necessary to determine the effects of prolonged exposure (McMichen et al. 2003). Thus, the degradation over time polymeric materials may increase can it could lead to gap formation, fluid infiltration (Kim et al. 2010) and leaching of monomers (McMichen et al. 2003). However, the incorporation of 10% amoxicillin-loaded microspheres did not alter the results of

softening in ethanol probably due to low water uptake of amoxicillin trihydrate, which favors its use compared to anhydrate or dehydrate (Tsuji et al. 1978; de la Torre et al. 2003).

In this study, addition of Amx-ME changed the rheological behavior. Small amounts of Amx-ME increased the viscosity in the resin-based sealer. However, the values of flow, as also for film thickness are in accord with those of the commercially available sealers, reported in previous studies and in accordance to ISO 6876:2001 (6876 2001; Versiani et al. 2006; Lee et al. 2011; Leitune et al. 2013). The results showed the important ability of experimental root canal sealer to create a thin film and adequate flowability, promoting a adequate filling of the root canal space (de Deus et al. 2003). The penetration of sealer inside dentinal tubules with animicrobial effect could prevent the bacteria growth in canal system (Razmi et al. 2010).

Citotoxicity resin-based sealers have different levels of cytotoxicity depending cells type and tested used Sulforhodamine B have been used due to its high sensitive and reproducibility. Cytotoxicity of resin-based sealers could be explained by methacrylate monomers leached from polymer matrix (Versiani et al. 2006; Al-Hiyasat et al. 2010). Methacrylate based commercial sealers had moderate and high cytotoxicity in fibroblast cells (Al-Hiyasat et al. 2010).

The rate of flare-up post-treatment endodontic ranges from 1.4 to 16% and its development is influenced mainly by microbial factors (Sipaviciute & Maneliene 2014). In cases of asymptomatic necrotic pulp, 47 to 60% had postoperative pain within the first 24 hours (Siqueira 2003). Not hermetic sealing of root canals, secondary infection and radiographic bone destruction

and extent of apical periodontitis are risk factors for the onset of postoperative pain and flare-up (Sipaviciute & Maneliene 2014). In this study, the experimental sealer showed a significant release of drug in the first 48 hours. This may be related to the antimicrobial activity in the same period.

Conclusion

Microspheres containing amoxicillin were successfully incorporated the experimental endodontic sealer. The addition of 10% of amoxicilin microspheres showed antimicrobial effect and high cell viability without changing the properties of sealer. Thus, the encapsulation method with slow release of drugs incorporated in sealers may be a therapeutic option in cases of primary and secondary infection.

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Tabela

Table 1. Mean (±standard deviation) of flow and thickness film, softening in solvent and degree of conversion of experimental sealer

Groups	flow (mm)	Film thickness (µm)	Softening in solvent			Degree of conversion	
			KHN1	KHN2	%ΔKHN	Immediate	24h
GC (0% Amx-ME)	26.83 (± 1.01)^{A}	20.00 (± 10.00)^{A}	23.22 (± 2.36)^{Aa}	10.56 (± 1.22)^{b}	54.5% (± 0.007)^{A}	51.73 (± 9.72)^{A*}	60.86 (± 6.79)^{A*}
G10 (10% Amx-ME)	21.53 (± 0.70)^{B}	26.66 (± 11.54)^{A}	21.70 (± 1.82)^{Aa}	9.88 (± 0.65)^{b}	54.4% (± 0.06)^{A}	55.13 (± 1.43)^{A*}	60.79 (± 6.93)^{A*}
G15 (15% Amx-ME)	23.55 (± 1.79)^{B}	26.66 (± 5.77)^{A}	24.38 (± 1.40)^{Aa}	10.66 (± 1.22)^{b}	56.2% (± 0.05)^{B}	52.70 (± 6.42)^{A*}	73.80 (± 3.43)^{A*}

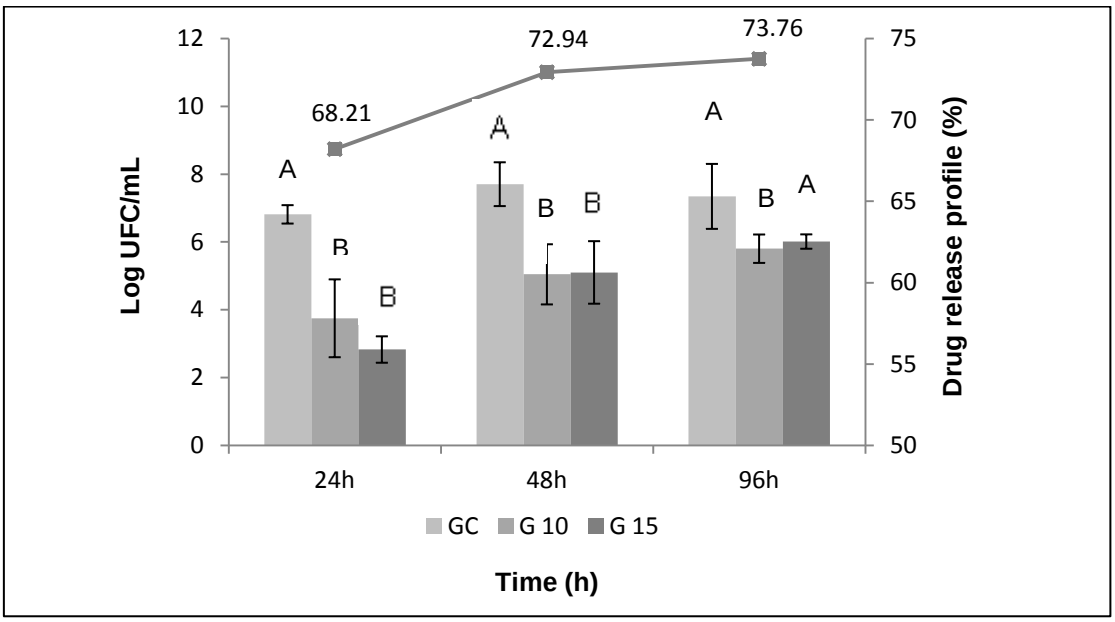
Different capital letters indicate statistical difference in the same column for all tests ($p < 0.05$

Different lowercase letters indicate statistical difference in the same line for softening in solvent test($p < 0.05$)

* Indicate that there was no statistical difference between the lines in the degree of conversion test

Figuras

Fig. 1. Release profile of AMX-ME from the experimental sealer in SBF immersion until 96 h and antimicrobial activity against *enterococcus faecalis* until 96 h.

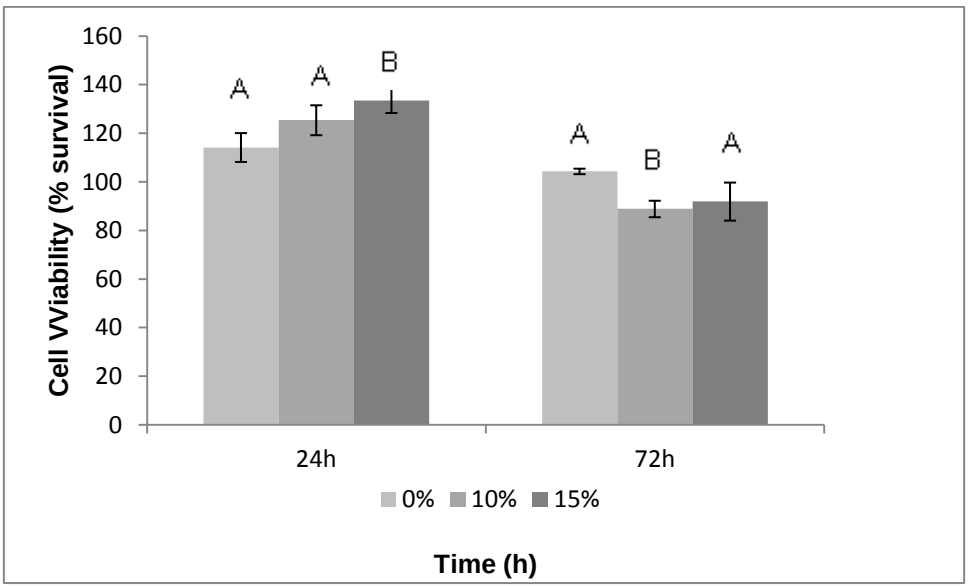


The above line in the graph show the drug release (in percent, %) versus time (hours). The columns show antimicrobial activity of the experimental groups against *enterococcus faecalis.* Log CFU / ml are shown against time (hours).

Different capital letters indicate statistical difference at antimicrobial test at the same time ($p < 0.05$)

Figura 2

Figure 2. Cytotoxicity assay of experimental endodontic sealer with dental pulp fibroblast at 24 and 72 hours eluate.



Different capital letters indicate statistical difference at the same time ($p < 0.05$)

4. Considerações finais

Novos cimentos endodônticos têm sido desenvolvidos e introduzidos no mercado odontológico ao longo dos últimos anos. O objetivo desses materiais é melhorar o selamento do sistema de canais radiculares, estimular reparos, melhorar as propriedades físico-químicas e apresentarem bioatividade.

O desenvolvimento de um cimento endodôntico que apresente atividade antimicrobiana contra *E. faecalis* é de grande importância. Este microrganismo esta associado em 32 a 70% dos casos de infecção após tratamento endoôntico (Wang *et al.*, 2012). Além disso, um cimento endodôntico que apresente baixa citotoxicidade, liberação lenta de um fármaco com ação antimicrobiana contra *E. faecalis,* pode ser uma alternativa terapêutica no tratamento não apenas dos casos de infecção secundária, mas também nos casos de infecção primária.

Os resultados obtidos a partir de testes *in vitro* realizados neste trabalho foram apresentados em forma de artigo e mostraram-se favoráveis a incorporação de microesferas contendo amoxicilina no cimento endodôntico experimental. Ainda assim, ensaios de radiopacidade, solubilidade e avaliação da interface cimento/dentina podem complementar os achados, principalmente, degradação hidrolítica ao longo do tempo e penetração/embricamento do cimento nos túbulos dentinários. características que se relacionam com a longevidade do material. Para a radiopacidade, considera-se um comportamento desejável de radiopacidade maior de 3mm de alumínio segundo a norma

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