



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba
Faculdade de Odontologia

Juliana de Carvalho Machado

**Efeito da combinação de antibióticos e sinvastatina sobre
microrganismos de interesse endodôntico e na expressão de
marcadores odontoblásticos**

Araçatuba – SP

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Mestre em Ciência Odontológica, Área Endodontia.

Orientadora: Prof^a. Dr^a. Cristiane Duque

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

Charles Chaplin

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Resumo

Terapias biológicas tem buscado novas substâncias/protocolos que promovam a eliminação microbiana e induzam ou estimulem a regeneração pulpar e o desenvolvimento completo radicular de dentes permanentes jovens com processos patológicos pulpares. Os objetivos do estudo foram avaliar a atividade antimicrobiana/antibiofilme de algumas combinações de antibióticos sobre microrganismos de interesse endodôntico e analisar o efeito da combinação de antibióticos com melhor ação antimicrobiana associada à sinvastatina na expressão de marcadores odontoblásticos em células da polpa dental humana (CPDH). A atividade antimicrobiana dos seguintes antibióticos: Metronidazol (ME), Ciprofloxacina (CI), Minociclina (MI), Doxicilina (DO) e Fosfomicina (FO), isolados ou em combinação dupla (ME+CI, ME+MI, ME+DO, ME+FO, CI+MI, CI+DO, CI+FO, DO+FO, MI+FO) ou tripla (ME+CI+MI, ME+CI+FO, ME+MI+FO, ME+CI+DO, ME+DO+FO, CI+DO+FO, CI+MI+FO) foram testados contra *Streptococcus mutans*, *Enterococcus faecalis*, *Actinomyces israelii* e *Candida albicans* em condições planctônicas. Biofilmes mono-espécie de *E. faecalis* e biofilmes em dual-espécies de *E. faecalis* and *C. albicans* foram preparados em blocos de dentina para testar a atividade antibiofilme das combinações de antibióticos com os melhores resultados microbiológicos. O efeito antibiofilme das combinações antibióticas sobre biofilme de *E. faecalis* dentro dos túbulos dentinários foi também avaliada por microscopia confocal. Culturas de CDPH foram expostas à combinação antibiótica com melhor resultado microbiológico e sinvastatina e determinada a viabilidade celular, atividade da fosfatase alcalina (ALP), deposição de nódulos de mineralização e expressão de DSPP (sialofosfoproteína dentinária), importante marcador odontoblástico de mineralização dentinária. Os dados foram

analisados estatisticamente, considerando $p < 0,05$. Todas as combinações de antibióticos reduziram o crescimento bacteriano, exceto por CI+DO e DO+FO para *A. israelii*. ME+CI+MI e ME+MI+FO inibiram significativamente o crescimento de *A. israelii* e *E. faecalis*, e ME+MI+FO eliminou *S. mutans*. ME+MI+FO e ME+CI+FO tiveram o melhor efeito contra biofilme de *E. faecalis*, em mono ou dual-espécies e dentro dos túbulos dentinários. CI e ME+CI+FO afetaram a viabilidade das células pulpares, em 1 e 7 dias. A atividade de ALP aumentou com a presença de sinvastatina para todos os grupos, exceto para CI e ME+CI+FO. Grupos contendo sinvastatina mostram maior deposição de nódulos de mineralização e expressão de DSPP que os grupos sem sinvastatina. Pode-se concluir que a combinação de antibióticos tripla ME+CI+FO teve efeito marcante contra os microrganismos endodônticos, em condições planctônicas e em biofilme. A sinvastatina estimulou a expressão de marcadores odontoblásticos de mineralização dentinária pelas HDPC; entretanto, seu efeito foi reduzido pela presença da CI.

Palavras-chave: antibacterianos, biofilme, técnicas de cultura de células, polpa dentária, sinvastatina

MACHADO, JC. Combined effect of antibiotics and simvastatin on endodontic microorganisms and the expression of odontoblast markers. Araçatuba, 2015.68p. Dissertação (Mestrado em Ciência Odontológica – Área de Endodontia) Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2016.

Abstract

Biological therapies have searching for substances/protocols, which promote microbial elimination and induce or stimulate pulp regeneration and completion of apical root development in young permanent teeth with pulp pathological processes. The objectives of this study were to evaluate the antimicrobial/anti-biofilm activity of some antibiotics combinations on endodontic microorganisms and the effect of the combination of antibiotics with the best antimicrobial action associated with simvastatin on expression of odontoblast markers by human dental pulp cells (HDPC). The antimicrobial activity of the following antibiotics: Metronidazole (ME), Ciprofloxacin (CI), Minocycline (MI), Doxycycline (DO) and Fosfomicin (FO), either alone or in double (ME+CI, ME+MI, ME+DO, ME+FO, CI+MI, CI+DO, CI+FO, DO+FO, MI+FO) or triple combinations (ME+CI+MI, ME+CI+FO, ME+MI+FO, ME+CI+DO, ME+DO+FO, CI+DO+FO, CI+MI+FO) were tested against *Streptococcus mutans*, *Enterococcus faecalis*, *Actinomyces israelii* and *Candida albicans* in planktonic conditions. Mono-species biofilm of *E. faecalis* and dual-species biofilms of *E. faecalis* and *C. albicans* were prepared in dentin blocks to test the anti-biofilm activity of antibiotic combinations with the best microbiological results. Antibiofilm effect of antibiotic combination on *E. faecalis* biofilm inside dentin tubules was also evaluated by confocal microscopy. Cultures of HDPC were exposed to the antibiotic combination with the best antimicrobial effect and simvastatin and determined cell viability, alkaline phosphatase activity, deposition of mineralization nodules and expression of *Dspp* (dentin sialophosphoprotein), important odontoblast markers of dentin mineralization. Data were analyzed statistically, considering $p < 0.05$. All antibiotic combinations reduced statistically the growth of bacteria tested, except by CI+DO and DO+FO for *A. israelii*. ME+CI+MI and ME+MI+FO inhibited significantly growth of *A.*

israelii and *E. faecalis*, and ME+MI+FO eliminated *S. mutans*. ME+MI+FO and ME+CI+FO had the best effect against *E. faecalis* biofilm, in mono and dual-species biofilms and inside dentin tubules, similar to CHX. CI and ME+CI+FO affected HDPC viability, 1 and 7 days. ALP activity increased with the presence of simvastatin for all groups, except by CI and ME+CI+FO. Groups containing simvastatin had higher mineralized nodule deposition and higher DSPP expression than groups without simvastatin. It can be concluded that triple antibiotic combination of ME+CI+FO had remarkable effect against endodontic microorganisms, in planktonic and biofilm conditions. Simvastatin stimulated the expression of odontoblast markers of dentin mineralization by HDPC; however, its effect was reduced in the presence of CI.

Keywords: anti-bacterial agents, biofilm, cell culture, dental pulp, simvastatin

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Lista de abreviaturas

MTA = Agregado de Trióxido Mineral

ME = Metronidazol

CI = Ciprofloxacina

MI = Minociclina

DO = Doxiciclina

SIM = Sinvastatina

CHX = Clorexidina

ANF = Anfotericina

MIC = Mínima Concentração Inibitória

MLC = Mínima Concentração Letal

ALP = Fosfatase Alcalina

HDPC = human dental pulp cells (células da polpa humana)

DSPP = Sialofosfoproteína dentinária

GAPDH = gliceralde- hude-3-phosphate dehydrogenase (gene endógeno)

CLSM = Microscopia Confocal de Varredura à Laser

DAP = Pasta Diantibiótica

TAP = Pasta Triantibiótica

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Introdução Geral

O tratamento de danos pulpares irreversíveis em dentes com rizogênese incompleta tem sido considerado um desafio clínico. Estes dentes, além de não apresentarem a formação completa da raiz, apresentam paredes finas, frágeis e divergentes que tornam o tratamento mais difícil e delicado (AL ANSARY *et al.*, 2009). Atualmente, as terapias endodônticas tradicionais para esses casos consistem na apicificação, que pode ser realizada de duas maneiras. A primeira utiliza um anteparo de MTA (Agregado de Trióxido Mineral) na porção apical, permitindo a obturação posterior com gutta-percha e a segunda, a mais utilizada, se refere às trocas periódicas de hidróxido de cálcio, como medicação intracanal até o fechamento apical (Rafter, 2005). Para ambas as formas de tratamento a instrumentação vigorosa é necessária, o que pode fragilizar as paredes dentinárias, além de eliminar qualquer remanescente celular no ápice radicular e a possibilidade de revascularização e, portanto, ausência do término da formação radicular. Além disso, o tratamento com hidróxido de cálcio envolve um grande tempo de trocas do medicamento até a formação de uma barreira apical, que tem se apresentado com consistência porosa e não contínua (SHAH *et al.*, 2008; IGLESIAS-LINARES *et al.*, 2013).

As técnicas de tratamento endodôntico atuais possibilitam uma redução substancial na quantidade e na diversidade de microrganismos presentes no interior dos canais radiculares. Porém, é importante considerar que para o preparo químico-mecânico, são indicadas substâncias químicas irrigadoras como o hipoclorito de sódio ou a clorexidina, que em concentrações antimicrobianas, não são consideradas biocompatíveis, podendo inviabilizar as células tronco presentes na região periapical e impedindo a aderência na superfície dentinária e sua proliferação para posterior secreção de dentina (RING *et al.*, 2008). Além disso, devido à complexa malha de canais secundários e acessórios e a persistência de algumas espécies microbianas, o preparo químico-mecânico não possibilita a total desinfecção do sistema de canais radiculares, levando à necessidade do uso de medicação intracanal (BYSTRÖM E SUNDQVIST, 1981; CHÁVEZ DE PAZ *et al.*, 2003). Entretanto, diversos autores têm detectado uma média de 5 espécies bacterianas, atingindo em torno de 10^2 a 10^5

células/canal, mesmo após o preparo químico-mecânico, seguido ou não de medicação intracanal (BYSTRÖM E SUNDQVIST, 1985; SJÖGREN *et al.*, 1997; VIANNA *et al.*, 2006; SAKAMOTO *et al.*, 2007; SIQUEIRA *et al.*, 2007a,b). Comparando esses dados com os obtidos para as infecções endodônticas primárias, nas quais são verificadas até 30 espécies bacterianas e contagem até 10^8 células por canal infectado (SIQUEIRA *et al.*, 2004b, SIQUEIRA E RÔÇAS, 2005b; VIANNA *et al.*, 2006b; SAKAMOTO *et al.*, 2007; SIQUEIRA *et al.*, 2007b), nota-se uma redução substancial da diversidade/nível microbiano, entretanto ainda há espécies que resistem após os tratamentos atuais.

Bactérias Gram-negativas, membros comuns das infecções primárias, são geralmente eliminadas após procedimento de tratamento químico-mecânico. Exceções podem incluir alguns bacilos anaeróbios como *Fusobacterium nucleatum*, *Prevotella species* e *Campylobacter rectus*, que estão entre as espécies encontradas em amostras após instrumentação/medicação. Entretanto, a maioria dos estudos tem revelado que as bactérias Gram-positivas são as mais frequentes nestes casos, incluindo estreptococos (*S. mitis*, *S. gordonii*, *S. anginosus*, *S. sanguinis*, *S. oralis*), *P. micra*, *Actinomyces species* (*A. israelii* e *A. odontolyticus*), *Propionibacterium species* (*P. acnes* e *P. propionicum*), *P. alactolyticus*, lactobacilos (*L. paracasei*, *L. acidophilus*), *Enterococcus faecalis*, entre outros (SIQUEIRA E RÔÇAS, 2009).

Antimicrobianos naturais ou sintéticos estão sendo empregados como alternativas às medicações intracanaís tradicionais, com o intuito de eliminar as bactérias e suas toxinas e não agredir os tecidos periapicais adjacentes, buscando a regeneração tecidual e a completa formação radicular apical em dentes imaturos (IGLESIAS-LINARES *et al.*, 2013; MORENO-HIDALGO *et al.*, 2014). O tratamento de regeneração/revascularização pulpar é uma abordagem alternativa biológica para o tratamento de dentes com comprometimento pulpar e rizogênese incompleta. Este tratamento, ao contrário da técnica de apicificação, permite a continuação do desenvolvimento da raiz (BÜYÜKBAYRAM *et al.*, 2014). As pesquisas na área de revascularização iniciaram nas décadas de 50 e 60 com estudos voltados para dentes reimplantados ou transplantados (MYERS E FLANAGAN, 1958; ÖSTBY, 1961). Estes estudos mostraram que para que ocorra a regeneração tecidual, é importante a formação do coágulo sanguíneo, pois este irá induzir a formação do tecido conectivo

fibroso no interior dos canais radiculares. Sendo assim, para o sucesso dos protocolos de revascularização é essencial a assepsia do canal radicular (ÖSTBY, 1961).

Há algumas teorias que buscam explicar o mecanismo da revascularização em dentes permanentes imaturos. Alguns autores acreditam que estes dentes apresentam células pulpares ainda vitais no ápice radicular e estas poderiam se diferenciar em odontoblastos por estímulo dos restos epiteliais de Mallassez e sintetizar dentina (BANCHS E TROPE, 2004). Outra teoria refere-se à sobrevivência de células indiferenciadas da polpa dental que poderiam se aderir às paredes internas do canal radicular, se diferenciando em odontoblastos e depositando dentina, terminando, assim, a formação do ápice radicular (GRONTHOS *et al.*, 2002). Uma terceira teoria estaria relacionada à presença de células indiferenciadas provenientes da papila apical ou da medula óssea no interior do canal radicular, que após a indução de sangramento na região periapical, poderiam proliferar e na presença de fatores de crescimento do coágulo, se diferenciar em células odontoblásticas sintetizadoras de dentina (WANG *et al.*, 2007).

A maioria dos protocolos de revascularização utiliza a irrigação passiva com hipoclorito de sódio associada à medicação intracanal com uma pasta composta de três antibióticos (THIBODEAU E TROPE, 2007; REYNOLDS *et al.*, 2009; KIM *et al.*, 2010; IWAYA *et al.*, 2011). O objetivo deste tratamento é criar um ambiente apropriado no interior do espaço do canal radicular, incluindo a ausência de bactérias e tecido necrótico pulpar e permitir a presença de uma estrutura de suporte e um vedamento coronal. Isso promove o repovoamento das células-tronco, a regeneração do tecido pulpar e a conclusão da formação do ápice radicular (BÜYÜKBAYRAM *et al.*, 2014).

Sato *et al.* (1993) e Hoshino *et al.* (1996) foram os primeiros investigadores a utilizarem a pasta triantibiótica contendo metronidazol, ciprofloxacina e minociclina. Hoshino *et al.* (1996) avaliaram a eficácia antibacteriana destes medicamentos isoladamente e em combinação contra bactérias de dentina infectada, polpas infectadas e periodontite apical e observaram que nenhum deles sozinho poderia eliminar todas as bactérias, mas em combinação eram suficientemente potentes para erradicar as bactérias em todas as amostras. Em 1996, Sato *et al.* observaram que esta mesma combinação foi eficaz contra bactérias em camadas profundas do canal radicular. Desde então, outros pesquisadores vem utilizando esta mesma associação

antibiótica em diversos estudos, em sua maioria de caráter clínico, como parte de protocolos de revascularização (BANCHS E TROPE, 2004; THIBODEAUD *et al.*, 2007; JUNG *et al.*, 2008; DING *et al.*, 2009; TANEJA *et al.*, 2010) alcançando bons resultados, com a eliminação das bactérias e promoção do reparo dos tecidos periapicais.

O metronidazol é um composto nitroimidazol que exibe ampla atividade antiprotozoária, antibacteriana contra bacilos e cocos anaeróbios. A ciprofloxacina é uma fluoroquinolona que apresenta ação bactericida, principalmente contra Gram-negativas. A minociclina é um derivado semi-sintético da tetraciclina com amplo espectro de ação, similar ao metronidazol (VIJYAGARAGHAVAN *et al.*, 2012). O uso da pasta contendo esses três antibióticos vem gerando algumas discussões com relação à resistência bacteriana, descoloração da coroa e possível reação alérgica (COHENCA *et al.*, 2010; KIM *et al.*, 2010; AKCAY *et al.*, 2014). Os genes de resistência antibiótica mais prevalentes detectados em amostras de infecções endodônticas crônicas foram os das tetraciclinas: tetM (42%) e tetW (29%). As tetraciclinas também podem causar manchamento dentário, devido à sua reação com o cálcio via quelação formando um complexo insolúvel (TANASE *et al.*, 1998). Assim, novas alternativas à minociclina têm sido estudadas para a pasta triantibiótica, como a fosfomicina, um potente inibidor da proteína MurA, envolvida na biogênese da parede celular, com amplo espectro de ação contra bactérias Gram-positivas e Gram-negativas (TROPE, 2010).

Embora a ação antimicrobiana seja uma propriedade primordial de uma medicação intracanal, é interessante que, nos casos de dentes permanentes com ápice aberto, essa medicação também tenha a capacidade de induzir células remanescentes a se diferenciarem em odontoblastos e a produzirem dentina interradicular para o término da formação radicular.

As estatinas são inibidores específicos da redutase da coenzima A 3-hydroxy-3-methylglutaryl, amplamente utilizadas como agente redutor de colesterol na prevenção e tratamento de aterosclerose. Elas apresentam certos efeitos adicionais, como a ação anti-inflamatória e aumento da função das células endoteliais vasculares e a angiogênese (KWAK *et al.*, 2000; SAKODA *et al.*, 2006). Vários estudos têm sido realizados para verificar os efeitos benéficos das estatinas, principalmente a sinvastatina, em Odontologia. Efeitos pós-cirúrgicos têm sido apontados em alguns estudos que utilizaram a sinvastatina, como o auxílio na remodelação óssea,

osseointegração em implantes de titânio e aumento do reparo de áreas de fraturas (LIU *et al.*, 2009; DU *et al.*, 2009; SALLAM *et al.*, 2011). Em Periodontia, a sinvastatina tem mostrado reduzir a perda óssea alveolar e a mobilidade dentária em casos de periodontite crônica (PRADEEP *et al.*, 2010; FAJARDO *et al.*, 2010). Em Endodontia, estudos têm mostrado efeito na regeneração dentinária e óssea, aumentando o crescimento celular e a diferenciação odontogênica (OKAMOTO *et al.*, 2009; LEE *et al.*, 2012). Varalskshmi *et al.* (2013) avaliaram a ação da sinvastatina e atorvastatina associadas ao carreador alpha-TCP (fosfato tricálcico) e verificaram que a atividade da fosfatase alcalina - ALP, a expressão de marcadores de mineralização (Sialofosfoproteína Dentinária (DSPP), Proteína da Matriz Dentinária-1 (DMP-1), Fosfatase Alcalina (ALP)) e a proliferação/diferenciação celular foi maior no grupo tratado com as estatinas que no grupo com MTA, indicando o efeito indutor de diferenciação de células pulpares desse material. Assim, os objetivos do estudo foram avaliar a atividade antimicrobiana/antibiofilme de algumas combinações de antibióticos sobre microrganismos de interesse endodôntico e analisar o efeito da combinação de antibióticos com melhor ação antimicrobiana associada à sinvastatina na expressão de marcadores odontoblásticos em células da polpa dental humana. Esse estudo pretende contribuir para a área de Endodontia com o estudo de novas alternativas biológicas que auxiliem na eliminação da infecção e favoreçam a regeneração tecidual de dentes permanentes imaturos.

ARTIGO

Combined effect of antibiotics and simvastatin on endodontic microorganisms and the expression of odontoblast markers by human dental pulp cells

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Abstract

Aims: To evaluate the antimicrobial/anti-biofilm activity of some antibiotic combinations on endodontic microorganisms and the effect of the combination of antibiotics with the best antimicrobial action associated with simvastatin on expression of odontoblast markers by Human Dental Pulp Cells (HDPC).

Methodology: Metronidazole (ME), Ciprofloxacin (CI), Minocycline (MI), Doxycycline (DO) and Fosfomycin (FO), either alone or in double or triple combinations were tested against *Streptococcus mutans*, *Enterococcus faecalis*, *Actinomyces israelii* and *Candida albicans* in planktonic conditions. Mono-species biofilm of *E. faecalis* and dual-species biofilms of *E. faecalis* and *C. albicans* were prepared in dentin blocks to test the anti-biofilm activity of antibiotic combinations with the best microbiological results. Anti-biofilm effect of antibiotic combination on *E. faecalis* biofilm inside dentin tubules was also evaluated by CLSM. Culture of HDPC were exposed to antibiotic combination and simvastatin and determined cell viability, alkaline phosphatase activity (ALP), deposition of mineralization nodules and expression of DSPP (Dentin Sialophosphoprotein), important odontoblast markers of dentin mineralization. Data were analyzed statistically, considering $p < 0.05$.

Results: All antibiotic combinations reduced statistically the growth of bacteria tested, except by CI+DO and DO+FO for *A. israelii*. ME+CI+MI and ME+MI+FO inhibited significantly growth of *A. israelii* and *E. faecalis*, and ME+MI+FO eliminated *S. mutans*. ME+MI+FO and ME+CI+FO had the best effect against *E. faecalis* biofilm, in mono and dual-species biofilms and inside dentin tubules, similar to CHX. CI and ME+CI+FO affected HDPC viability, at 1 and 7 days. ALP activity increased with the presence of simvastatin for all groups, except by CI and ME+CI+FO. Groups containing simvastatin had higher mineralized nodule deposition and higher DSPP expression than groups without simvastatin.

Conclusion: Triple antibiotic combination of ME+CI+FO had remarkable effect against endodontic microorganisms, in planktonic and biofilm conditions. Simvastatin stimulated the expression of odontoblast markers of dentin mineralization by HDPC; however, its effect was reduced in the presence of ciprofloxacin.

Introduction

The treatment of irreversible damages to dental pulp of immature permanent teeth caused by trauma or infection before physiological apical closure is considered a clinical challenge. Besides the large apical opening, these teeth have thin divergent or parallel dentinal walls which difficult conventional endodontic treatment, leading to extrusion of irrigants and medicaments beyond the foramen (Al Ansary *et al.* 2009). The standard therapeutic approach consists on use of calcium hydroxide or Mineral Trioxide Aggregate - MTA to induce apexification or the apical closure by forming a hard tissue barrier across the root apex. However, the apexification does not result in continued root development because none of these materials stimulates pulp revascularization. Then the interruption of the radicular formation reduces the resistance of the dentin walls and increases the risk of root fracture (Iglesias-Linares *et al.* 2013).

The chemical-mechanical preparation by conventional endodontic treatments lead to a substantial reduction in the quantity and diversity of microorganisms, however, does not allow full disinfection of the root canal system and persistent microorganisms, frequently Gram-positive facultative bacteria including enterococci and streptococci, have been recovered at the end of the treatment or between appointments (Chávez de Paz *et al.* 2003). Besides, common irrigating solutions, such as sodium hypochlorite and chlorhexidine gluconate, are cytotoxic to dental pulp stem cells (DPSC) preventing their attachment to root canal surfaces and subsequent secretion of dentin (Ring *et al.* 2008).

Biological therapies have searching for substances/protocols which promote microbial elimination and induce or stimulate pulp regeneration and completion of apical root development in pathological situations (Moreno-Hidalgo *et al.* 2014). Recent clinical discoveries have showed that apexogenesis is possible for both vital and non-vital pulp (Banchs & Trope 2004, Thibodeaud *et al.* 2007, Trope 2010) as a result of the induction of remaining DPSC or recruitment of new cells from area of revascularization in the periapex, enabling the root to develop in its full width and length (Iglesias-Linares *et al.* 2013).

Revascularization protocols include a passive irrigation with sodium hypochlorite associated with an intracanal dressing composed of three antibiotics,

metronidazole, ciprofloxacin and minocyclin (Banchs & Trope 2004). This treatment intended to create an appropriate environment inside root canal space, without viable bacteria and allow the proliferation of stem cells provided from remaining vital pulp tissue and Hertwig's epithelial root sheath. A blood clot is created after disinfection which acts a matrix for the growth of new tissue into the pulp space (Banchs & Trope 2004). However, the use of triple antibiotic paste has generated some discussion regarding bacterial resistance, crown discoloration and possible allergic reaction (Kim et al. 2010, Akcay et al. 2014). Another question is whether to use these three antibiotics as originally reported, or in an improved form, using a mixture of other combinations (Iglesias-Linares *et al.* 2013).

Although antimicrobial activity is a primordial property of a canal dressing, the association with a substance able to induce cells to differentiate into odontoblasts and produce interradicular dentin could be ideal for the treatment of immature permanent teeth. Statins are specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase widely used as cholesterol-reducing agents in the prevention and treatment of atherosclerosis. Studies have showed that statins exert anti-inflammatory action, enhance the function of vascular endothelial cells and angiogenesis and could induce dentin and bone regeneration (Kwak *et al.* 2000, Varalskshmi *et al.* 2013). The objectives of this study were to evaluate the antimicrobial activity of some antibiotic combinations on endodontic microorganisms and the effect of the combination of antibiotics with the best antimicrobial action associated with simvastatin on the expression of odontoblast markers by human dental pulp cells.

Material and methods

Preparation of antibiotics, simvastatin and controls

The antibiotics tested were: Metronidazole (ME), Ciprofloxacin (CI), Minocycline (MI), Doxycycline (DO) and Fosfomycin (FO). The choice of antimicrobial agents was based on the studies of Hoshino *et al.* (1996) and Trope (2010). Antibiotics were weighed with an analytical balance (OHAUS Adventurer, Parsippany, NY, USA) at 4mg/mL and dissolved in sterile deionized water. Simvastatin (SIM) was dissolved in sterile deionized water at 20µM. Chlorhexidine digluconate (CHX) were dissolved in sterile deionized water to a concentration of 20mg/mL and used as positive control. All

solutions were filtered using 0.22µm syringe filters (Kasvi, Curitiba, PR, Brazil) and stored at -20°C or +4°C, following the manufacturer's recommendations. Antibiotics, simvastatin and CHX were purchased from Sigma-Aldrich (St. Louis, MO, USA). Groups of the study are present in Table 1.

Table 1. Antibiotic combinations and controls.

Antibiotic combinations					
G1: ME	G5: FO	G9: ME + FO	G13: DO + FO	G17: ME + MI + FO	G21: CI + MI + FO
G2: CI	G6: ME + CI	G10: CI + MI	G14: MI + FO	G18: ME + CI + DO	G22: CHX
G3: MI	G7: ME + MI	G11: CI + DO	G15: ME + CI + MI	G19: ME + DO + FO	G23: distilled H ₂ O
G4: DO	G8: ME + DO	G12: CI + FO	G16: ME + CI + FO	G20: CI + DO + FO	

Antimicrobial activity

Microbial strains and growth conditions

The following standard strains were used: facultative anaerobic bacteria - *Enterococcus faecalis* (ATCC 51299), *Streptococcus mutans* (ATCC 25175), *Actinomyces israelii* (ATCC 12102) and the aerobic yeast, *Candida albicans* (ATCC 26790). All strains were provided by Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil). The purity of the strains was confirmed by the Gram's method. Microbial suspensions were prepared from cultures previously grown in Mitis Salivarius Agar (Difco Laboratories, Kansas City, MO, USA) with 0.2U/mL bacitracin (Sigma-Aldrich) for *Streptococcus mutans*, Brain Heart Infusion Agar – BHIA (Difco Laboratories) for *A. israelii* and *E. faecalis* and incubated at 37°C for 24h in a 5% CO₂ atmosphere (Incubator Ultra Safe, HF212-UV). *C. albicans* was cultured in Sabouraud Dextrose Agar (SDA) (Difco Laboratories) containing 40mg/mL chloramphenicol (Sigma-Aldrich) at 37°C in aerobic conditions for 24h. For subsequent microbiological assays, standard optical density was established for each microorganism: 0.5 for bacteria (approximately 1-5x10⁸ CFU/mL) and 0.3 for *C. albicans* (approximately 1-5x10⁶ CFU/mL) at 550nm. The absorbance was measured using a microplate reader (Eon

Microplate Spectrophotometer, BioTek Instruments, USA) to assess the microorganism density.

Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were obtained using the microdilution method with 96-wells microtiter plates, based on the criteria of the National Committee for Clinical Laboratory Standards (according to NCCLS M27-A2) for yeast and the National Committee for Clinical Laboratory Standards (according to NCCLS M7-A5) for bacteria with some modifications. After reactivation of *S. mutans*, *A. israelii* and *E. faecalis* in solid medium, 5-10 representative colonies were cultured in BHI broth at 37°C for 24 h in 5% CO₂. Colonies of *C. albicans* were cultured aerobically in RPMI-1640 medium (Sigma-Aldrich) under agitation at 135rpm and 35°C. Microbial cultures were grown until reaching the standard optical density - OD (described on previously) and harvested by centrifugation (Hanil Combi centrifuge, 514R) at 4000xg for 10min. The supernatant was discarded and the pellet re-suspended in 2x concentrated Mueller-Hinton broth (MH, Difco Laboratories) for bacteria and 2x concentrated RPMI-1640 medium (Sigma-Aldrich) for *C. albicans*. The final concentration of bacterial suspension inside the wells was 1-5x10⁵CFU/mL and 1-5x10³CFU/mL for *C. albicans*. Chlorhexidine digluconate (CHX) was used as positive control and cultures without antimicrobial agents were used as negative control. All antimicrobial agents were serially diluted in sterile deionized water to obtain the concentrations ranging from 0.03µg/mL to 2000µg/mL. The microbial suspensions were inoculated in each well containing the previously diluted antibiotics/controls. The microplates were incubated at 37°C for 24h for all microorganisms. Afterwards, 15µL of 0.01% resazurin (Sigma-Aldrich) was applied in each well and incubated for 4h to determine cell viability. After incubation, the last blue well (MIC) and at least three previous wells were serially diluted and plated on Mueller-Hinton Agar (MHA) for bacteria and SDA for *C. albicans* for 48h at the same conditions. After that, viable bacteria were counted and the number of Colonies Forming Units/mL (CFU/mL) was determined. The minimal lethal concentration (MLC)

was obtained when the antimicrobial agents killed more than 50% of the tested microbial strains.

Based on the MIC results obtained in the tests with the antibiotics isolated, dual and triple antibiotic associations were prepared with a maximum volume of 30µL inside wells containing a total of 200µL of microbial culture plus antibiotics. The microbial cultures were placed into wells containing the antibiotic associations and incubated for 24h. Afterwards, wells were stained with resazurin for 4h. Aliquots from resultant blue wells were diluted, plated and incubated at 37°C for 48h and after that, CFU/mL counts was determined. All experiments were performed in triplicate in three independent experiments (n=9).

Anti-biofilm activity

Dentin block preparation

This part of the study was approved by the Ethical Committee in Animal Experimentation of Araçatuba Dental School, UNESP, Brazil (Protocol: 2014/00595 – ANEXO B). Bovine incisors were extracted and stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. Initially, roots were separated from crowns, 1 mm below the cement-enamel junction, using a diamond disc (KG Sorensen D 91, Barueri, SP, Brazil). In order to obtain slices of dentin, roots were fixed to acrylic plates and sectioned transversally with two diamond discs (Extec Diamond Wafer Blade, series 12205, 102 mm x 0.3 mm x 12.7 mm, Extec Corporation, Enfield, CT, USA) using a precision saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA) separated by a spacer disk (thickness: 3 mm) under irrigation with deionized water. Root dentin slices were cut in four blocks with the following dimensions: 3 mm x 3 mm x 0.7 mm. Dentin blocks (n = 10) were sequentially polished using 400-, 600-, 800- and 1200-grit water-cooled silicon carbide paper disks (Carbimet Paper Disks, Buehler) in a polisher (Vector Power Head Buehler). The dentin blocks were washed with distilled water and ultrasonically cleaned using 17% EDTA for 3min and deionized water for 5min. After autoclaving for 15 minutes at 121°C, the dentin blocks were stored at 4°C until use within one week (Liu *et al.* 2010).

Biofilm assays

Mono-species biofilm of *E. faecalis* and dual-species biofilms of *E. faecalis* and *C. albicans* were prepared to test the anti-biofilm activity of the triple antibiotic combinations with the best results on MIC/MLC assays. Initially, dentin blocks (n = 30) were fixed with double sided tape to the bottom of 96-wells microplates. Initially, 100µL of bacterial suspension (or 50µL for each microorganism in dual-species biofilm) in BHI with 1% glucose were inoculated in each well in a final concentration of $1-5 \times 10^7$ CFU/mL. Blocks were randomized divided in two groups: Initial biofilm (n=15), in which antibiotic combination was placed at the beginning of the experiment (1h after insertion of the microbial suspensions into wells) to determine the inhibitory effect of antibiotics on the growth of biofilm and Mature biofilm (n=15), in which antibiotic combination was keep in contact with biofilm previously formed for 48h, in order to study the inhibitory effect of antibiotics on mature biofilm. After 48h of incubation at 37°C in 5% CO₂, bacterial suspensions were aspirated and each dentin block was washed once with 100µl of 0.9% saline. For mature biofilm, antibiotics were incubated for 24h. After the periods of incubation, each dentin block was washed twice with 100µL of 0.9% saline and aseptically removed from the wells and incubated for 1h into microtubes containing 500µL of Cystein-Peptone (5g yeast extract, 1g peptone, 8.5g NaCl, 0.5g L-cysteine HCl and 100 mL of glycerine per liter, pH. 7.3) at 37°C to inactivate the action of antibiotics (Deng *et al.* 2004). For recovery of bacterial cells within the dentinal tubules, the blocks were submitted to an ultrasonic bath using deionized water for 20min, followed by vortexing (AP 56, Phoenix) for 2min. Aliquots were serially diluted and plated in M-Enterococcus Agar (Difco) for *E. faecalis* and SDA with choramphenicol for *C. albicans*. The plates were then incubated for 48h and the number of CFU/mL was calculated. The experiments were performed in triplicate in three independent assays (Li *et al.* 2012).

Confocal Laser Scanning Microscopy (CLSM)

Biofilm assays for CLSM analysis were conducted with *E. faecalis* testing the triple antibiotic combinations with the best results on MIC/MLC assays. For this analysis, bovine roots were prepared according to methodology proposed by Ma *et al.* (2011) with some modifications. Briefly, bovine roots (n=3/group) were sectioned

horizontally 1mm below the cemento-enamel junction to obtain a dentin block with a length of 4mm. Root canals were enlarged with Gates Glidden drill#6 (1.5mm diameter) (Dentsply) at 300rpm at low speed. Each cylindrical dentin block was fractured into two semi cylindrical halves. The outer surfaces of halves were ground by 600-grit silicon carbide paper to achieve a standard thickness of 5mm. The end block size was 4mm x 4mm x 5mm.

The blocks were cleaned in ultrasonic bath using initially 17% EDTA solution for 3 minutes and distilled water for 5 minutes. After cleaning, the blocks were dried, sterilized and inserted in a microtube with the canal side up. Gaps between the dentin block and inner wall of tube were sealed by resin composite (3M) and light cured for 40 seconds. Five hundred microliters of *E. faecalis* suspension at 10^7 CFU/mL in BHI broth was added to each microtube with dentin blocks inside. The microtubes were harvested at 1400g, 2000g, 3600g and 5600g in a sequence twice each for 5 minutes and a fresh solution of bacteria was inserted between every centrifugation and the last solution was discarded.

Dentin blocks were incubated individually in 24-wells plates in BHI broth for 15 days, replacing culture medium every 48h. After this period, blocks were washed twice with sterile saline and transferred, under aseptic conditions, to a new plate and exposed to antibiotic combinations at 5X MLC concentration for 24h. After that, dentin blocks were washed twice again and cut into transverse slices of 1mm thickness using a precision cutter and polished with 1200-grit sandpaper disks. After new washing with sterile water, blocks were stained with 100 μ L of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide, according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500nm for SYTO 9 and 490/635nm for propidium iodide. Two additional uninfected specimens were stained using the same protocol as negative controls. Fluorescence from the stained cells was viewed using CLSM (Leica TCS SP5, Microsystems GmbH) with a 40x oil immersion lens. CLSM images were acquired using software (LAS AF Leica Microsystems) at a resolution of 1024 by 1024 pixels. Ten-micrometer-deep scans (0.2 μ m step size, from the outer to inner dentin) were obtained from two randomly selected places with the CLSM. In order to analyze the Live/Dead cell ratios of the infected dentinal tubules, all scan were

reconstructed in a three-dimensional model using the same software. The quantification of red fluorescence ratio in relation to green-and-red fluorescence was determined using COMSTAT software, indicating the proportion of dead cells for each antibiotic combination tested (Li *et al.* 2012).

Expression of mineralization markers by human dental pulp cells (HDPCs)

Cell cultures

HDPCs were obtained by enzymatic digestion of pulp tissue from freshly impacted third molar surgically extracted and donated by a young patient (Certificate of Presentation for Ethical Consideration approved by the Human Research Committee of UNESP, Brazil – CAAE #41736714.7.000.5420 – ANEXO C). The pulp tissue was incubated with type II collagenase (200 units/mL; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 24h at 37°C and 5% CO₂. Thereafter, the cells were subcultured in complete DMEM (Dulbecco's Modified Eagle Medium; supplemented with 100 IU/mL penicillin, 100mg/mL streptomycin, 2mmol/Lglutamine; Gibco, Grand Island, NY, USA) and 10% heat-inactivated FBS (Fetal Bovine Serum; Gibco). Cells at passage #3 were used in this experiment.

Experimental procedures

For experimental analysis, the cells were seeded on 96-wells plates to 80% confluence (5×10^3 cells/well). HDPCs were exposed to the best antibiotics (ME+CI+FO) at the concentration of 5x MLC, either alone or in combination. All antibiotics/combinations were also associated with simvastatin 0.1µM/L (Sigma Aldrich) (Okamoto *et al.* 2009).

Cell viability assays

Cell viability was assessed by the Alamar Blue assay after 1 and 7 days in culture. At each time-point (n=6), the cells were incubated with DMEM plus Alamar Blue dye (10:1) for 4h at 37°C, and the fluorescence intensity was monitored at 530nm excitation and 590nm emission (Synergy H1, BioTek, Winooski, VT, USA). The cell viability was calculated considering NC group as 100% of cell viability at day 1. The

increase/decrease in cell viability percentage was used for indirect determination of cell proliferation throughout the periods of analysis (Soares *et al.* 2015a).

Alkaline phosphatase (ALP) activity

Pulp cells (5×10^3 cells/well) were seeded in cell culture 96 wells plates and pre-incubated in DMEM plus 10% SFB for 48 hours. After 14 days of incubation in osteogenic culture medium (DMEM plus 10% SFB, supplemented with 10 nmol/L β -glycerophosphate and 50 μ g/mL sodium ascorbate; Sigma-Aldrich Corp.) containing the combinations of antibiotics and simvastatin, replace every 72h, cells were disrupted in 0.1% sodium lauryl sulphate (Sigma Aldrich). ALP activity in the supernatant was determined by assessing the release of thymolphthalein by hydrolysis of thymolphthalein monophosphate substrate using a commercial kit (Labtest, Lagoa Santa, Minas Gerais, Brazil), following the manufacturer's instructions. In all the tubes were added 50 μ L of substrate and 0.5 mL of diethanolamine buffer, 0.3 mmol/mL, pH 10.1. In standard tube was added 50 μ L of the standard solution. The tubes are kept at 37°C for 2 minutes. In each tube, 50 μ L of the lysed cells after stimulation with combinations of antibiotics/simvastatin were also added. The tubes were kept at 37°C for 10 minutes. After this period, 2 ml color reagent (0.09 mmol Na_2CO_3 /mL and 0,25 mmol NaOH/mL) was placed in each tube and then the absorbance measured in a spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, California, USA) at a 590 nm wavelength. The alkaline phosphatase activity was converted into U/L by means a standard curve with known amounts of ALP. Total protein dosage was performed for normalization of ALP, as previously described by Soares *et al.* (2015a), and absorbance was measured at a 655 nm wavelength. The absorbance values obtained was converted into mg/L by a standard protein curve. The final value of ALP was normalized by total protein data and the ALP activity (U/mg) was transformed into a percentage with the mean value of negative control considered as presenting 100% of ALP activity (Soares *et al.* 2015a). Two independent experiments were performed.

Alizarin red staining

Pulp cells (5×10^3 cells/well) were seeded in cell culture 96 wells plates and pre-incubated in medium for 48 hours. After 21 days incubation in osteogenic culture

medium containing the combinations of antibiotics and simvastatin, replaced every 72h, the cells (n=6) were washed with PBS, fixed in 70% ethanol at 4°C, and then stained with alizarin red dye (40mM, pH 4.2; Sigma) for 20min with shaking (VDR Shaker, Biomixer, Ribeirão Preto, SP, Brazil). After aspiration of unincorporated dye, the cells were washed twice with deionized water for the removal of excess stain, and representative images from each group were taken by light microscopy (Olympus BX51, Olympus, Miami, FL, USA).

Analysis of DSPP gene expression by real-time PCR

Immediately after washing in PBS solution, the cells were incubated in osteogenic medium containing the combination of antibiotics with simvastatin and controls for 21 days, which was replaced every 72h. At the end of the experimental period, total RNA was extracted with an RNAqueous[®]-micro kit (Ambion, Austin, TX, USA), and 2µg was then reverse-transcribed into single-stranded cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's protocols. The gene expression of Dentin Sialophosphoprotein (DSPP) was assessed by real-time PCR with TaqMan assays DSPP, Hs00171962_ml and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), Hs02758991_g1. The qPCR reactions were prepared with standard reagents (TaqMan Master Mix, Applied Biosystems, Life Technologies, Warrington, UK) and performed using a Step One Plus Thermocycler (Applied Biosystems) and CT values analyzed by Step One Plus Software (Soares *et al.* 2015b). The CT values for each sample were normalized by the endogenous control gene, and then data was normalized by negative control group.

Statistical analysis

Antimicrobial activity of double and triple combinations in planktonic conditions and anti-biofilm effect of triple combination of antibiotics on mono-species biofilm and dual-species biofilm formed in dentin blocks were determined based in CFU/mL counts obtained for each microorganism tested and converted in Log (CFU/mL +1). These data were submitted to ANOVA and Tukey tests. Box-plots were performed to represent the quantification of dead cells in relation to total cells (dead/live+dead

cells) in dentin blocks exposed to triple combinations of antibiotics. CLSM data were submitted to Kruskal-Wallis and Mann-Whitney tests. Data from cell viability were expressed in means/standard deviations and submitted to ANOVA/Tukey tests. Percentage of ALP activity and mRNA levels of Dspp gene for HDPC cells were represented in box-plots and submitted to Kruskal-Wallis and Mann-Whitney tests. Statistical significant was considered when $p < 0.05$.

Results

Antimicrobial activity

MIC and MLC assays

Among the antibiotics, doxycycline (DO) and minocycline (MI) have the best bactericidal activity for both times of exposure, showing MIC values ranging from 0.03 μ g/mL to 0.24 μ g/mL and MLC values ranging from 0.06 to 0.48 μ g/mL. Ciprofloxacin (CI) had remarkable antimicrobial action on *S. mutans*, *A. israelii* and *E. faecalis* with low MIC/MLC values ranging: 1.95 to 62.5 μ g/mL). Fosfomycin (FO) also present inhibitory effect against bacterial tested, however lower than tetracyclines and ciprofloxacin. Metronidazole (ME) presented antimicrobial effect on *A. israelii* and *S. mutans* at high concentrations, but not on *E. faecalis*. *Candida albicans* growth was affected by tetracyclines, at high concentrations, and CHX. Simvastatin had no effect against the microorganisms tested, at 10 μ M, 100x concentration that will be used for cell culture assays. CHX had antimicrobial effects similar to tetracyclines with very low values of MIC/MLC ranging from 0.24 to 4.89 μ g/mL (Table 2).

Effect of double and triple antibiotic combination in planktonic conditions

Considering double combinations of antibiotics, CI+FO was the only one that eliminated all bacteria tested. All antibiotic combinations reduced statistically the growth of bacteria tested, except by CI+DO and DO+FO for *A. israelii*. After CI+FO, the combinations ME+CI, ME+MI, ME+DO were the most effective against three bacteria, completely eliminating *S. mutans*. DO+FO and MI+FO also eliminated *S. mutans*, but were not effective for *A. israelii* (Figure 1). For triple combinations, ME+CI+FO reduced significantly *A. israelii* growth and eliminated *S. mutans* and *E. faecalis*. ME+CI+MI and

ME+MI+FO also inhibited significantly growth of *A. israelii* and *E. faecalis*, and ME+MI+FO eliminated *S. mutans* (Figure 2).

Table 2. Minimal inhibitory concentration (MIC) and Minimal lethal concentration (MLC) values obtained by antibiotics and controls after 24 h of exposure, in planktonic conditions.

	MIC (MLC) µg/ml			
	<i>E. faecalis</i>	<i>A. israelii</i>	<i>S. mutans</i>	<i>C. albicans</i>
Metronidazole (ME)	> 2000	500 (500)	500 (1000)	-
Ciprofloxacin (CI)	15 (62.5)	1.95 (3.90)	15 (31)	-
Fosfomycin (FO)	31.25 (62.5)	1.95 (7.80)	62.5 (125)	-
Doxycycline (DO)	0.12 (0.24)	0.03 (0.06)	0.24 (0.48)	500 (> 2000)
Minocycline (MI)	0.06 (0.48)	0.06 (0.12)	0.06 (0.48)	1000 (1000)
Chlorhexidine (CHX)	4.89 (4.89)	0.98 (0.98)	0.24 (7.81)	39 (39)
Simvastatin (SIM)	> 10µM	> 10µM	> 10µM	> 10µM

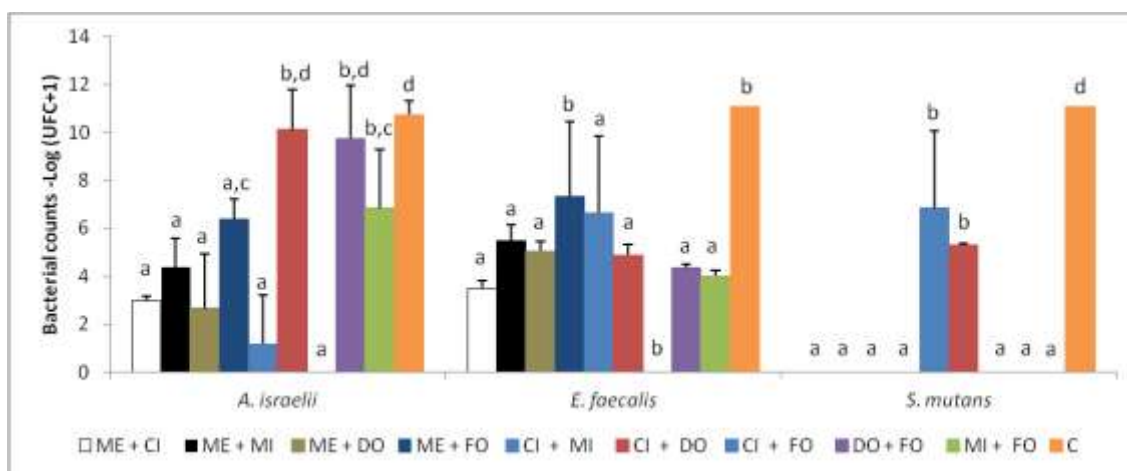


Figure 1. Antimicrobial activity of double combinations of antibiotics after 24h of exposure, in planktonic conditions.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering each bacterial species separately, according to ANOVA/Tukey tests.

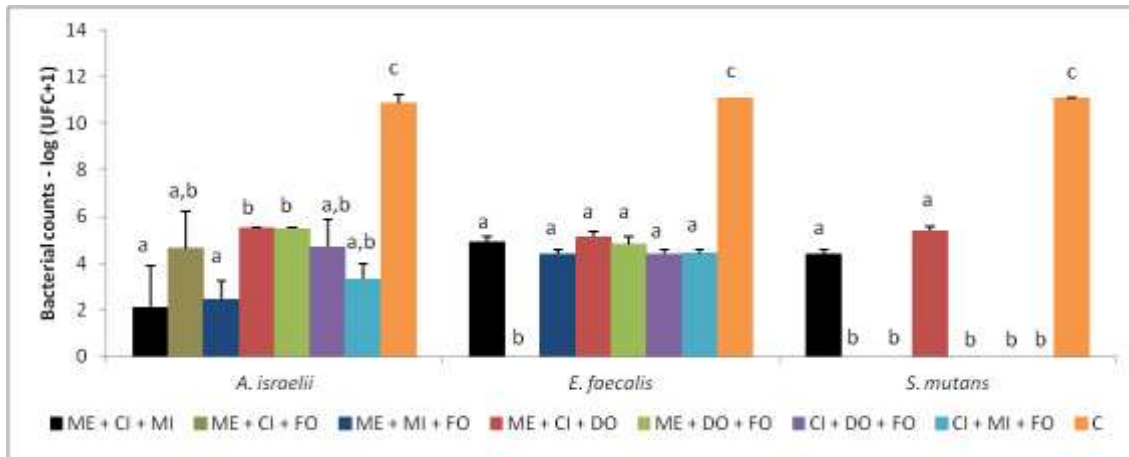


Figure 2. Antimicrobial activity of triple combinations of antibiotics after 24h of exposure, in planktonic conditions.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering each bacterial species separately, according to ANOVA/Tukey tests.

Anti-biofilm activity

In mono-species biofilm, combinations of antibiotics tested (ME+CI+MI, ME+CI+FO and ME+MI+FO) at 5X MLC eliminated *E. faecalis* when incorporated to culture medium at the initial stage of biofilm. For mature biofilm, the same combinations significantly reduced *E. faecalis* growth (Figure 3 A). In dual-species biofilm, ME+CI+FO and ME+MI+FO eliminated *E. faecalis* at the initial biofilm and reduced *E. faecalis* in mature biofilm (Figure 3 B). *C. albicans* counts were significantly reduced by antibiotics combinations in initial and mature biofilms (Figure 3 C). CHX 5x MLC eliminated or reduced *E. faecalis* and reduced *C. albicans* on both mono-species and dual-species biofilms. Among the triple antibiotic combinations, CLSM results showed that ME+MI+FO and ME+CI+FO had the best effect against *E. faecalis* biofilm inside dentin tubules, similar to CHX (Figure 4).

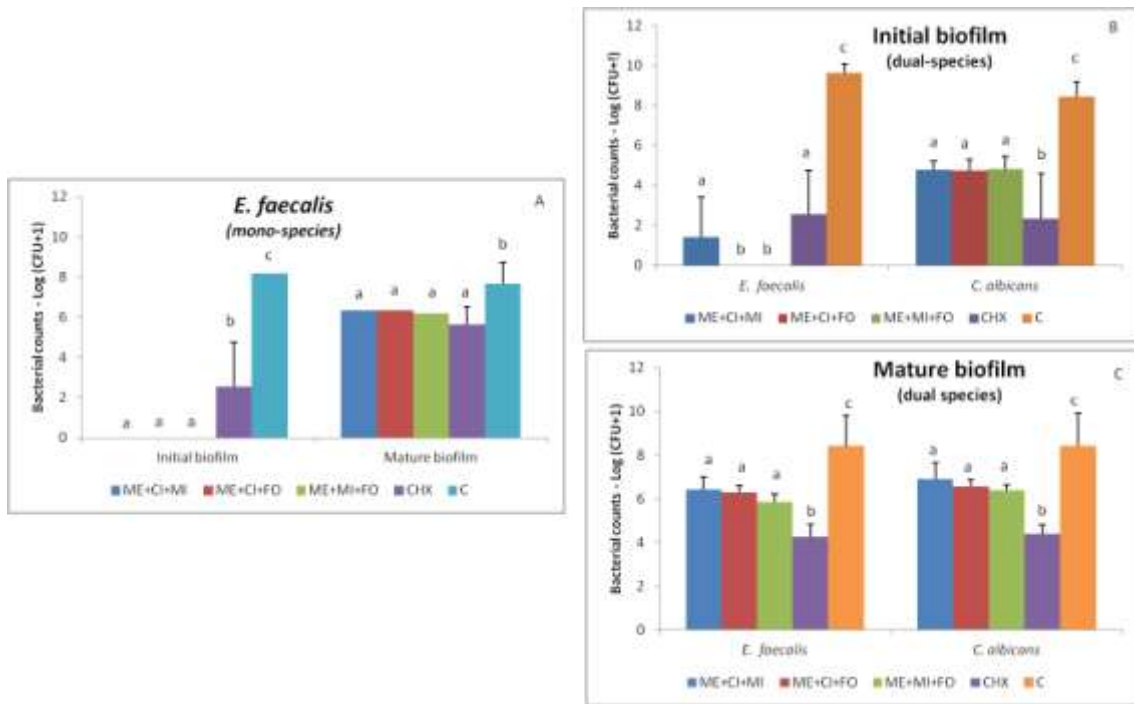


Figure 3. Effect of triple combination of antibiotics on mono-species biofilm (*E. faecalis*) and dual-species biofilm (*E. faecalis* and *C. albicans*) formed in dentin slices.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering each bacterial species separately, according to ANOVA/Tukey tests.

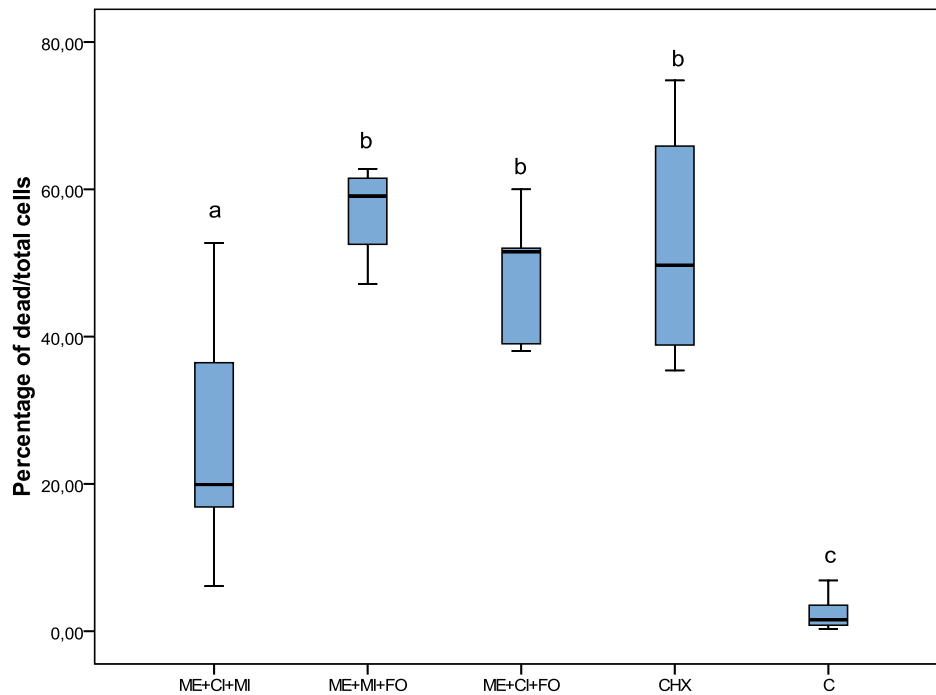


Figure 4. Effect of antibiotic in triple combination on *E. faecalis* biofilm formed inside dentin tubules, observed by confocal microscopy.

^a Different lower case letters show statistical difference among the groups of antibiotics, according to Kruskal-Wallis and Mann-Whitney tests.

Expression of odontoblast markers by human dental pulp cells (HDPCs)

Cl and ME+Cl+FO affected HDPC viability, independent of the presence of simvastatin, 1 and 7 days after antibiotic exposure. FO and ME did not affect cell viability at the concentrations tested (5X MLC). Comparing 1day to 7 days, cell growth significantly increased even in the presence of antibiotics and ME+Cl+FO combination, but percentage of cell viability was lower for Cl and ME+Cl+FO groups (Figure 5). ALP activity of HDPCs increased with the presence of simvastatin for all groups, except by Cl and ME+Cl+FO (Figure 6). Representative areas of HDPC culture in the wells, after alizarin red staining, showed that groups containing simvastatin had higher mineralized nodule deposition than groups without simvastatin (Figure 7). The expression of *Dspp*, an important odontoblast marker of dentin deposition, was increased with the presence of simvastatin, except by Cl. Comparing groups with SIM, all

groups had lower mRNA values than control (culture medium). CI, FO and ME+CI+FO had similar *Dspp* expression results, significantly lower than ME (Figure 8).

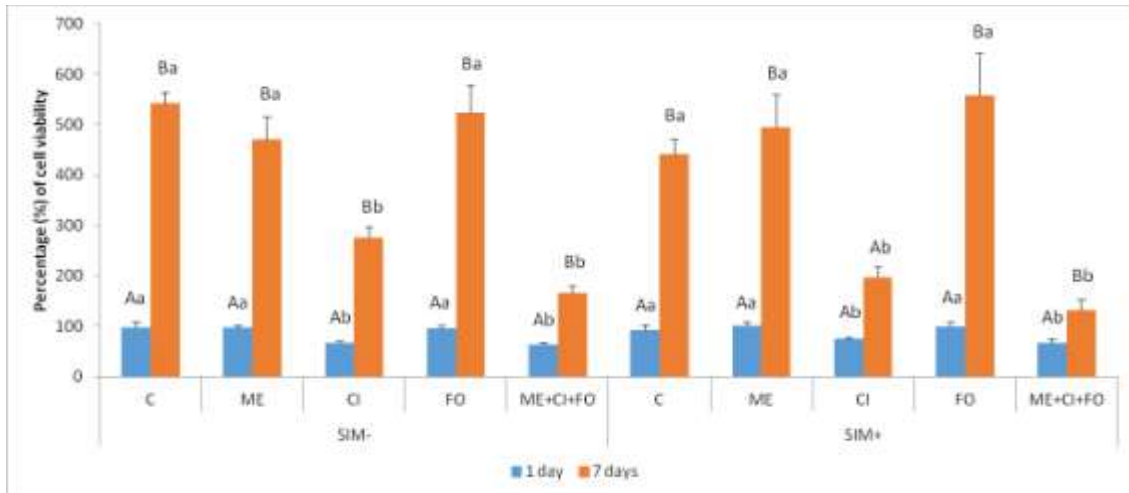


Figure 5. HDPC viability after 1 and 7 days of antibiotic exposure, using Alamar blue assays.

^A Different upper case letters show statistical difference between times of exposure (1 and 7 days), considering each antibiotic group separately, according to ANOVA and Tukey tests.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering each time of exposure (1 and 7 days) separately, according to ANOVA and Tukey tests.

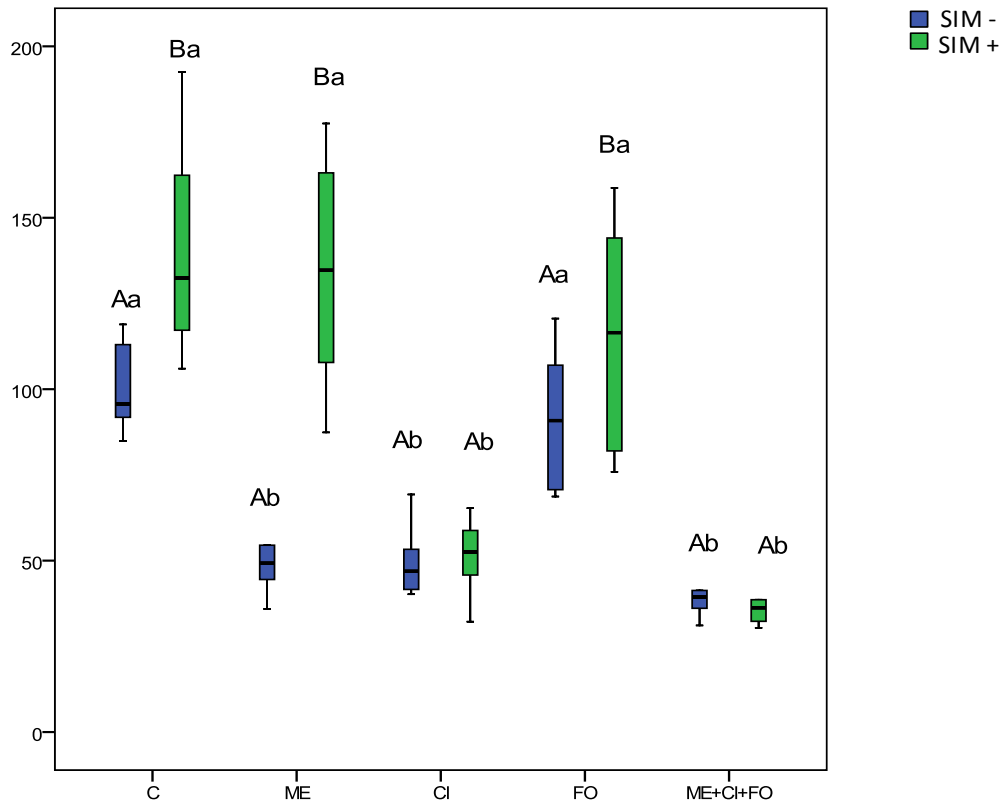


Figure 6. Percentage of ALP activity of HDPC for each group of antibiotic containing or not simvastatin (SIM -or SIM+), after 14 days of exposure.

^A Different upper case letters show statistical difference between the groups containing or not simvastatin (SIM- or SIM+), considering each antibiotic group separately, according to Kruskal-Wallis and Mann-Whitney tests.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering the presence of simvastatin (SIM- or SIM+) separately, according to Kruskal-Wallis and Mann-Whitney tests.

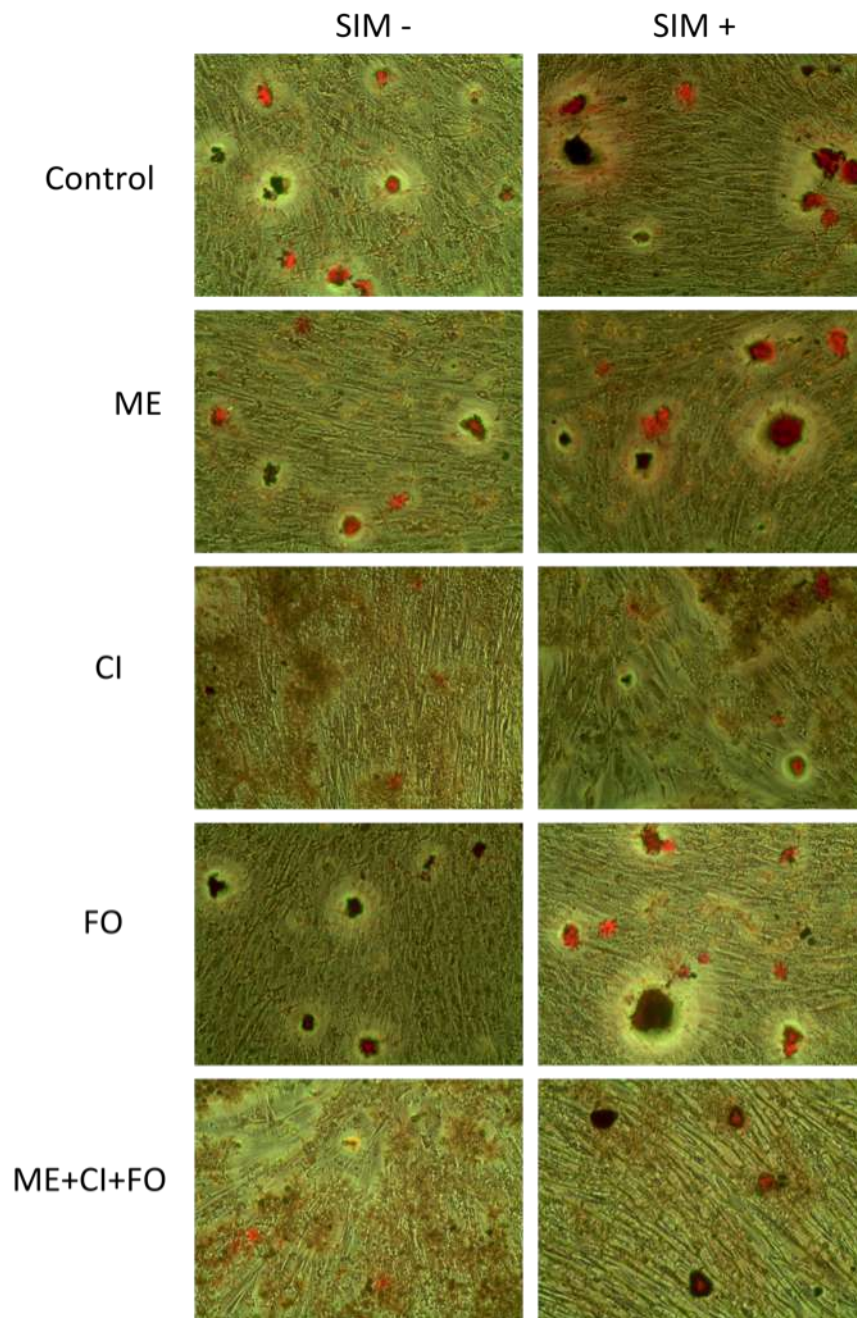


Figure 7. Alizarin red assays. Panel of mineralized nodule deposition for each group of antibiotic containing or not simvastatin (SIM- or SIM+) after 21 days of exposure. Light-microscopy images (10X) are from representative areas of HDPC in the wells. Groups with CI presented the lowest levels of nodule deposition.

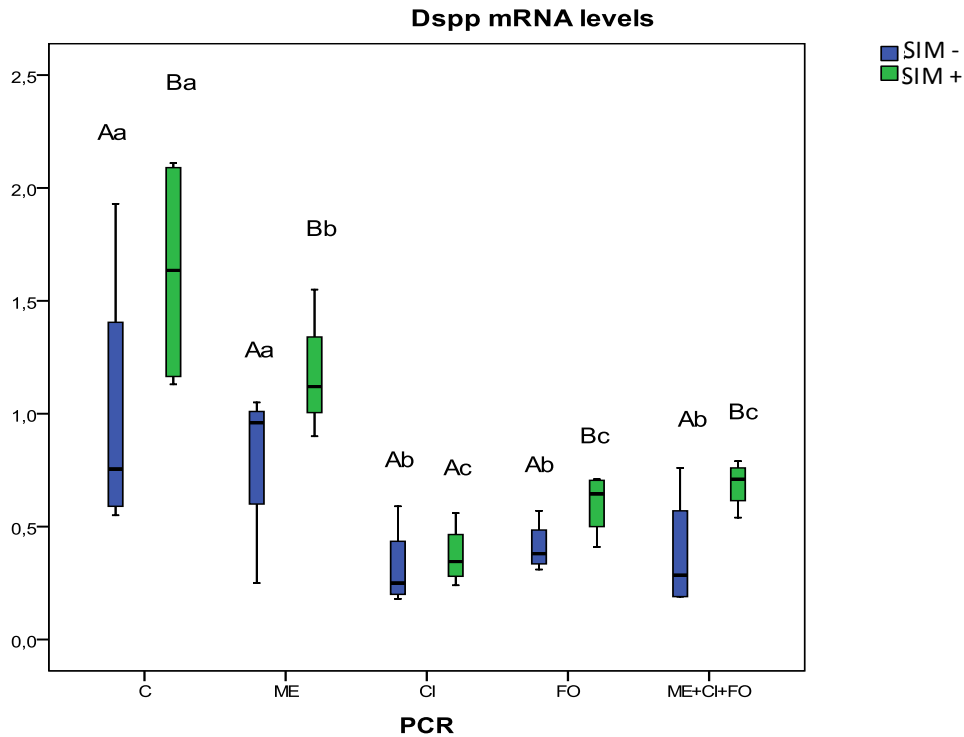


Figure 8. PCR assays. Levels of mRNA for *Dspg* gene of HDPC after 21 days of antibiotic exposure in the presence or not of simvastatin (SIM-or SIM+).

^A Different upper case letters show statistical difference between groups containing or not simvastatin (SIM- or SIM+), considering each antibiotic separately, according to Kruskal-Wallis and Mann-Whitney tests.

^a Different lower case letters show statistical difference among the groups of antibiotics, considering the presence of simvastatin (SIM- or SIM+) separately, according to Kruskal-Wallis and Mann-Whitney tests.

Discussion

Even after chemical-mechanical treatment, bacteria can be found inside the complex systems of accessory and lateral root canals. Among them, Gram-positive species, such as *Enterococcus* spp, *Actinomyces* spp. and *Streptococcus* spp, are most often associated with failed root canal therapy (Chávez de Paz *et al.* 2003). Systemic antibiotic therapy is not recommended for the treatment of periradicular lesions. However, antibiotics in the form of a final rinse, paste, or other releasing devices may be useful in eliminating residual bacteria and lead to healing of pulpar or periapical diseases (Byström *et al.* 1985). In this present study, five antibiotics, Metronidazole (ME), Ciprofloxacin (CI), Fosfomicin (FO), Doxycycline (DO) and Minocycline (MI) and the control chlorhexidine (CHX) were tested against *E. faecalis*, *A. israelii*, *S. mutans* and *C. albicans*. The choice of antimicrobial agents was based on the studies of Hoshino *et al.* (1996) and Trope (2010).

In this present study, the antibiotics DO and MI have the best bactericidal activity, followed by CI and FO against the species tested. ME presented antimicrobial effect on *A. israelii* and *S. mutans* at high concentrations, but not on *E. faecalis*. Similar results for DO (MIC ranging from 0.06 to 0.25 µg/mL) (Smith *et al.* 2005, LeCorn *et al.* 2007) but not for CI (MIC₉₀ > 32µg/mL) were found against several strains of *A. israelii* (Smith *et al.* 2005). Classical study showed MIC ranging 0.06 – 1 µg/mL for MI and 0.12 – 2 µg/ml for DO against 31 strains of *A. israelii* (Lerner, 1974). Fosfomicin showed activity against planktonic and adherent *E. faecalis*, with MIC value of 32µg/mL, similar to that found in the present study (31.25µg/mL). Lower values of MIC for CI (0.2µg/mL) and ME (0.2µg/mL) were found for *E. faecalis*, different from our study (Kaushik *et al.* 2015). In the present study, ME was ineffective against *E. faecalis* and MIC was extremely high for *A. israelii* and *S. mutans*. ME was also inactive against any of the *Actinomyces* spp. tested (MIC₉₀ = 256 µg/mL) in the study of LeCorn *et al.* (2007). The detection of *Candida* spp., mainly *C. albicans*, from infections resistant to conservative root canal therapy have attracted attention for the role of this yeast in endodontic pathologies (Waltimo *et al.* 2013). For this reason, *C. albicans* was included as a coadjuvant pathogen for biofilm analysis. Interestingly, *C. albicans* growth was affected by tetracyclines at high concentrations, but these concentrations were not

considered for the experiments of the study because of the cytotoxicity. Positive control chlorhexidine inhibited *C. albicans* at low concentrations, as expected.

In this current study, the most effective double combination against three bacteria was CI+FO, the only one that eliminated the three bacteria tested, followed by ME+CI, ME+MI, ME+DO. Synergic effect was observed when fosfomycin was combined with ampicillin, linezolid, minocycline, rifampicin, tigecycline, teicoplanin, vancomycin, particularly with teicoplanin against 89% of vancomycin-resistant *E. faecalis* (Tang *et al.* 2013). Ciprofloxacin when combined with fosfomycin and vancomycin was indifferent against two strains of *E. faecalis* in the log phase after at concentration ranging 0.5 – 10x MIC, differing from our results (Zuccarelli *et al.* 1989). Double antibiotic paste (DAP, a ME+CI mixture) has been studied as alternative to triple antibiotic paste (TAP, ME+CI+MI) and it has been reported that as low as 0.001 mg/mL of DAP can inhibit (MIC) the growth of *E. faecalis* and *Porphyromonas gingivalis* (Sabrah *et al.* 2013).

Although double combination of CI+FO was effective against all bacteria, we chosen to test triple combinations, including ME. The combination of ME, a antibiotic with broad bactericidal spectrum against anaerobes, with another antibiotics is still needed because endodontic infections generally involve multiple species, and only three bacteria were investigated in this study and none of them was strict anaerobic bacteria. Among triple combinations, ME+CI+FO, ME+CI+MI and ME+MI+FO significantly inhibited bacterial growth in planktonic conditions. These combinations also eliminated initial biofilm or reduced mature biofilm of *E. faecalis*, either mono-species or dual-species with *C. albicans*. The triantibiotic paste (TAP), proposed by Hoshino *et al.* (1996) is composed by combination ME+CI+MI and the most of studies is focused in the laboratorial or clinical evaluation of this mixture. Study showed that TAP completely eliminated *E. faecalis* biofilms at concentrations of 10 to 100 mg/mL, whereas 0.01 to 1 mg/mL TAP resulted in significant reduction of *E. faecalis* counts compared with saline control group for 1 or 4-week treatment (Reyhani *et al.* 2015). Case-report studies have demonstrated good clinical results using TAP for permanent teeth, with bacterial reduction and healing of periapical tissues (Banchs & Trope 2004, Thibodeaud *et al.* 2007, Trope 2010), however, the presence of minocycline resulted in tooth discoloration (Kim *et al.* 2010). MI seems to acts through the chelation of

calcium ions, forming calcium-enriched insoluble agglomerates over the dentin surface, as demonstrated by Porter *et al.* (2015) by energy-dispersive x-ray spectroscopy analyses. Besides, in primary molars, current studies have shown that non-instrumentation endodontic treatment using TAP showed good clinical success but had a low success rate based on radiographic evaluation at long-term follow-up (Prabhakar *et al.* 2008, Trairatvorakul & Detsomboonrat 2012). In the present study, we used 5X MLC for each antibiotic, considering that high unnecessary concentrations could be toxic to host cells (Ruparel *et al.* 2012). Another studies have been incorporated ME+CI+MI with less than 1g/mL concentration (TAP concentration) in different vehicles, such as gels and scaffolds, and demonstrated significant anti-biofilm effect against *E. faecalis*, *P. gingivalis* and *A. naeslundii* (Algarni *et al.* 2008, Ordinola-Zapata *et al.* 2013, Albuquerque *et al.* 2015).

The antibiotic combinations, ME+CI+MI, ME+CI+FO and ME+MI+FO, were effective against dual-species of biofilm, reducing or eliminating *E. faecalis* and *C. albicans* in initial or mature biofilm. Similar results were found by Shaik *et al.* (2014) that evaluated four medicaments: TAP+saline, TAP+chitosan, Ca(OH)₂ +salina and Ca(OH)₂+chitosan, and all of them showed antifungal and antibacterial activity, but the groups with chitosan had higher effect than saline, probably because chitosan also have antimicrobial effect. In our study, confocal scanning laser microscopy (CSLM) showed that ME+CI+FO and ME+MI+FO had superior *E. faecalis* antibiofilm effect inside dentin tubules than ME+CI+MI (TAP composition). Some investigators showed that TAP solution eliminated biofilm of *A. naeslundii* from root canals (Albuquerque *et al.* 2015) and reduced bacteria-mixed biofilm more than Ca(OH)₂ paste and 2% CHX gel (Ordinola-Zapata *et al.* 2013), both using CLSM.

Statins are structural analogs of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A). The association of statin with antibiotics could promote both antimicrobial effect and induction of stem cells to differentiate into odontoblasts and produce interradicular dentin in cases of pulp pathologies. In this study, we chosen the antibiotic combination with the best antimicrobial and anti-biofilm effects, ME+CI+FO, and mixed with simvastatin 0.1μM. The concentration of simvastatin was based in the study of Okamoto *et al.* (2009) that tested 0.1, 1 and 10μM and observed that when simvastatin was added to the culture of human pulp stem cells, the proliferation was

significantly increased using 0.1 μM after 1, 3 and 5 days of exposure. Simvastatin at 10 μM suppressed stem cells in day-3 and at 1 and 10 μM in day-5 cultures. The same study showed that simvastatin at 0.1 and 1 μM induced larger amount of mineralized tissue and DSPP gene expression up to 3.7 times significantly in comparison to the control. In the current study, we tested simvastatin at 0.1 μM , either alone or combined with antibiotics. Simvastatin was not toxic to pulp cells and induced ALP activity, mineralized nodule deposition and increased the DSPP gene expression, confirming that simvastatin accelerates the differentiation of pulp stem cells and induce dentin formation.

Previous study evaluated the effect of different concentrations of triantibiotic combination (ME+CI+MI) and recommended that a safe concentration is in the range of 0.39mg/mL and 1mg/mL because higher concentrations may limit tissue regeneration (Chuensombat *et al.* 2013). However, another study showed 100% stem cell death after 7 days of treatment, in the 1-mg/mL triantibiotic group. Even at 0.39mg/mL, the proliferative capacity and mineralized nodule formation of stem cells was significantly lower than that of untreated cells. Expression of genes related to dentin mineralization: DSPP, ALP and DMP-1 (Dentin Matrix Protein - 1) were similar to control groups (Karaxha *et al.*, 2013). In the present study, we chosen the antibiotic combination that has the best antimicrobial/anti-biofilm activities – ME+CI+FO, however, ciprofloxacin caused toxicity to pulp cells and interfered with ALP activity and mineralized nodule formation. Overall, DSPP expression was lower when antibiotics either alone or in combination were associated with simvastatin in comparison with the control groups. Further studies are necessary to test another antibiotic combinations that allow the induction of odontoblast markers by simvastatin.

Conclusion

Triple antibiotic combination of ME+CI+FO had remarkable effect against endodontic microorganisms, in planktonic and biofilm conditions. Simvastatin stimulated the expression of odontoblast markers of dentin mineralization by pulp cells; however, its effect was reduced in the presence of ciprofloxacin.

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Author Guidelines

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5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

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Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2.

Structure

All manuscripts submitted to *International Endodontic Journal* should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate

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Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

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- **Results:** Give the main results of the study, including the outcome of any statistical analysis.
- **Conclusions:** State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 words giving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250 words, including a clear research question, details of the literature search strategy and clear conclusions.

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- **Summary:** Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.

- **Key learning points:** Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

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- **Methodology:** Describe the methods adopted.
- **Results:** Give the main results of the study.
- **Conclusions:** State the primary conclusions of the study.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

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Conclusion: should contain a summary of the findings.

Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented.

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5.3.

References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

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In the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by *et al.* is sufficient, e.g. (Tobias *et al.* 1991). If more than 1 paper is cited the references should be in year order and separated by ", " e.g. (Pitt Ford & Roberts 1990, Tobias *et al.* 1991).

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- (II) Year of publication in parentheses
- (III) Full title of paper followed by a full stop (.)
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Examples of correct forms of reference follow:

Standard journal article
Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8-14.

Corporate author
British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* **16**, 192-5.

Journal supplement
Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* **54** (Suppl. 1), 26a.

Books and other monographs

Personal author(s)
Gutmann J, Harrison JW (1991) *Surgical Endodontics*, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a book
Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. *Endodontics in Clinical Practice*, 3rd edn; pp. 186-223. London, UK: Butterworth.

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DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of*

the Third Annual Meeting of the International Society for Experimental Rematology; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

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publication

Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States-1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8

Dissertation

or

thesis

Saunders EM (1988) In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee.

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Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text. Smith A (1999) Select committee report into social care in the community [WWW document]. URL <http://www.dhss.gov.uk/reports/report015285.html> [accessed on 7 November 2003]

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FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado "Efeito da combinação de antibióticos e sinvastatina sobre microrganismos de interesse endodôntico e na expressão de marcadores odontoblásticos", Processo FOA nº 2014-00595, sob responsabilidade de Cristiane Duque apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 26 de junho de 2014.

VALIDADE DESTE CERTIFICADO: 15 de Dezembro de 2016.

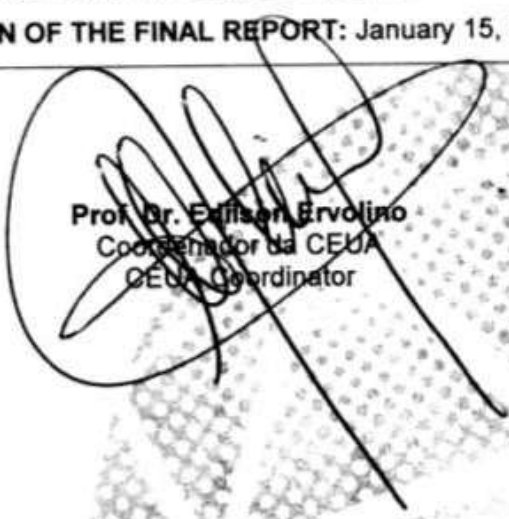
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 15 de Janeiro de 2017.

CERTIFICATE

We certify that the study entitled "Effect of combination of antibiotics and sinvastatin on endodontic microorganisms and its influence on the expression of odontoblast markers", Protocol FOA nº 2014-00595, under the supervision of Cristiane Duque presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on June 26, 2014.

VALIDITY OF THIS CERTIFICATE: December 15, 2016.

DATE OF SUBMISSION OF THE FINAL REPORT: January 15, 2017.



Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA Coordinator

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da combinação de antibióticos e sinvastatina sobre microrganismos de interesse endodôntico e na expressão de marcadores odontoblasticos.

Pesquisador: Cristiane Duque

Área Temática:

Versão: 2

CAAE: 41736714.7.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.119.112

Data da Relatoria: 12/06/2015

Apresentação do Projeto:

Com o intuito de eliminar as bactérias e suas toxinas sem agredir os tecidos periapicais adjacentes, favorecer a regeneração tecidual e a completa formação radicular apical em dentes permanentes imaturos, novas alternativas às medicações intracanais tradicionais estão sendo avaliadas. Alguns estudos, na sua maioria casos clínicos, utilizaram a pasta triantibiótica contendo metronidazol, ciprofloxacina e minociclina e obtiveram bons resultados clínicos, relacionados à capacidade antimicrobiana da pasta. Entretanto, além da ação bactericida da medicação intracanal, seria interessante a inclusão de um agente indutor da diferenciação de células indiferenciadas remanescentes, como a sinvastatina, para estimular a continuidade da formação radicular. Assim, o objetivo deste estudo é avaliar a ação antimicrobiana de antibióticos isolados ou combinados sobre bactérias de interesse endodôntico, em cultura planctônica e em biofilme e determinar o efeito das melhores combinações desses antibióticos associados com sinvastatina sobre a expressão de genes relacionados com o processo de mineralização da dentina. Os antibióticos, metronidazol, ciprofloxacina, minociclina, doxiciclina e fosfomicina, isolados ou combinados serão testados para avaliar sua atividade antimicrobiana sobre as seguintes cepas padrão: Enterococcus faecalis, Streptococcus mutans, Actinomyces israelii e Candida albicans. Serão realizados ensaios em cultura planctônica para determinar a mínima concentração inibitória (MIC) e mínima

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concentração bactericida (MBC) de cada antibiótico/combinção. Biofilmes de *E. faecalis* e *C. albicans* serão expostos a MBC das melhores combinações de antibióticos. Para avaliação do efeito da associação da sinvastatina aos antibióticos sobre a mineralização dentinária, culturas de células pulpares serão expostas a MBC dos peptídeos e analisadas as expressões dos genes DSPP e DMP-1 por qPCR, atividade da fosfatase alcalina e produção de nódulos mineralizados. Os dados obtidos serão analisados em programa estatístico, considerando $p < 0,05$. Este trabalho pretende contribuir para a área de Endodontia com o estudo de novas alternativas biológicas que auxiliem na eliminação da infecção e favoreçam a regeneração tecidual de dentes permanentes imaturos.

Objetivo da Pesquisa:

Verificar o efeito das melhores combinações de antibióticos associada à sinvastatina na citotoxicidade de células pulpares e a capacidade de induzir a expressão de marcadores de mineralização dentinária. Avaliação da expressão de marcadores genéticos relacionados com a mineralização dentinária; avaliação da viabilidade celular; avaliação da atividade da fosfatase alcalina; Análise da expressão de genes relacionados com a mineralização dentinária por PCR em tempo real.

Avaliação dos Riscos e Benefícios:

esta pesquisa visa somente a coleta de dentes previamente extraídos na clínica de Cirurgia por equipe especializada, portanto, não realizaremos os procedimentos cirúrgicos. Assim, a participação nesta pesquisa não ocasionará nenhum risco ou desconforto e a doação será completamente voluntária. Também não infringe as normas legais e éticas. Os procedimentos adotados nesta pesquisa obedecem aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução nº. 466/12 do Conselho Nacional de Saúde. Nenhum dos procedimentos usados oferece riscos à sua dignidade.

Benefícios:

Encontrar através das análises propostas, a menor concentração antibiótica capaz de, quando associada à sinvastatina, ser o menos citotóxica possível e induzir o término da formação radicular

Comentários e Considerações sobre a Pesquisa:

A pesquisa inicia-se a partir da doação de Dentes hígidos humanos recentemente extraídos e obtidos na Clínica de Cirurgia da Faculdade de Odontologia de Araçatuba –FOA/UNESP. Sob condições assépticas, os dentes serão abertos para remoção da polpa dentária com auxílio de limas endodônticas e serão ressuspensos, colocados em frascos para cultura.

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Considerações sobre os Termos de apresentação obrigatória:

a pesquisadora não corrigiu no protocolo (informações básicas do projeto) sobre riscos e benefícios. (é importante corrigir para risco mínimo)

De acordo com a resolução 466/12, item V – DOS RISCOS E BENEFÍCIOS; não importa que seja material orgânico humano descartado, o risco não é só físico mas existe a possibilidade de utilização e manipulação de informações genéticas que este tecido (dente-polpa) possui.

Portanto é estabelecido que:

Toda pesquisa com seres humanos envolve risco em tipos e gradações variados.

No sub-item V.2 - São admissíveis pesquisas cujos benefícios a seus participantes forem exclusivamente indiretos, desde que consideradas as dimensões física, psíquica, moral, intelectual, social, cultural ou espiritual desses.

no TCLE a pesquisadora descreve que não existe risco a integridade física e que estará realizando a pesquisa de acordo com as resoluções.

Recomendações:

a maior parte foram corrigidas

Conclusões ou Pendências e Lista de Inadequações:

n.d.n.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/12/2015.

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FACULDADE DE
ODONTOLOGIA - CÂMPUS DE
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Continuação do Parecer: 1.119.112

ARACATUBA, 23 de Junho de 2015

Assinado por:

Ana Claudia de Melo Stevanato Nakamune
(Coordenador)

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