Karina Sampaio Caiaffa

EFEITO CITOTÓXICO E ANTIMICROBIANO DE ANÁLOGOS DE PEPTÍDEOS CATIÔNICOS E SUA INFLUÊNCIA NA EXPRESSÃO DE MARCADORES FENOTÍPICOS E GENOTÍPICOS DE MINERALIZAÇÃO DENTINÁRIA

Araçatuba – SP
2015

Karina Sampaio Caiaffa

EFEITO CITOTÓXICO E ANTIMICROBIANO DE ANÁLOGOS DE PEPTÍDEOS CATIÔNICOS E SUA INFLUÊNCIA NA EXPRESSÃO DE MARCADORES FENOTÍPICOS E GENOTÍPICOS DE MINERALIZAÇÃO DENTINÁRIA

Dissertação de Mestrado apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Mestre em Ciência Odontológica - Área de Concentração: Endodontia.

Orientadora: Profª Drª Cristiane Duque

Araçatuba - SP
2015

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Caiaffa, Karina Sampaio.

C133e Efeito citotóxico e antimicrobiano de análogos de peptídeos catiônicos e sua influência na expressão de marcadores fenotípicos e genotípicos de mineralização dentinária / Karina Sampaio Caiaffa. - Araçatuba, 2015

132 f. : il. ; tab. + 1 CD-ROM

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba

Orientadora: Profa. Cristiane Duque

1. Peptídeos catiônicos antimicrobianos 2. Biofilmes 3. Expressão gênica I. Título

Black D24

CDD 617.67

Dados Curriculares

Karina Sampaio Caiaffa

Nascimento	02.06.1989 – Petrópolis - RJ
Filiação	Gerson da Silva Caiaffa Irani Sampaio Caiaffa
2008/2013	Curso de Graduação em Odontologia pela Faculdade de Odontologia da Universidade Federal Fluminense – Polo Universitário de Nova Friburgo, FOUFF/NF.
2010/2012	Desenvolvimento de Projeto de Iniciação Científica, com auxílio do Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.
2013/2014	Desenvolvimento de Projeto de Mestrado com auxílio do Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.
2014/2015	Desenvolvimento de Projeto de Mestrado com auxílio da Fundação de Amparo à Pesquisa do Estado de São Paulo.
Associações	CROSP - Conselho Regional de Odontologia de São Paulo. SBPqO - Sociedade Brasileira de Pesquisa Odontológica.

COMISSÃO EXAMINADORA

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE

Profa. Dra. Cristiane Duque – Orientadora. Professora Assistente do Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

Prof. Dr. Rodrigo Alex Arthur - Professor Adjunto II do Departamento de Ondontologia Social e Preventiva, Disciplina de Bioquimica e Microbilogia Bucais e Cariologia/Dentistíca da Faculdade de Odontologia, UFRGS - Universidade Federal do Rio Grande do Sul, Porto Alegre.

Prof. Dr. Rogério de Castilho Jacinto - Professor Adjunto do Departamento de Odontologia Restauradora, Disciplina de Endodontia da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

Membros suplentes:

Profa. Dra. Elisa Maria Aparecida Giro - Professora Adjunta do Departamento de Clínica Infantil, Disciplina de Odontopediatria da Faculdade de Odontologia - Araraquara, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araraquara.

Prof. Dr. Luciano Tavares Angelo Cintra - Professor Adjunto do Departamento de Odontologia Restauradora, Disciplina de Endodontia da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

"Feliz aquele que transfere o que sabe e aprende o que ensina." (Cora Coralina)

Dedicatória

Karina Sampaio Caiaffa

Dedico este trabalho,

Aos meus pais *Irani* e *Paulo*, *Gerson* e *Ana*, e a minha querida professora *Cristiane Duque*.

Por estarem comigo em todos os momentos, principalmente os mais difíceis, me apoiando e dando força para continuar... Pelo exemplo de simplicidade, dedicação e carinho. Agradeço por todos os momentos que estiveram juntos comigo e por todas as palavras de conforto e de incentivo que me mostraram que os momentos mais felizes que passei foram aqueles que tive força para lutar.

Sem vocês, esse dia tão especial em minha vida não estaria se realizando, pois muitas vezes o cansaço e a vontade de desistir surgiam, e foram nesses momentos que me lembrava de não estar sozinha, e que existiam pessoas que confiavam em mim;

Meus amores, vocês são tudo para mim!

Amo vocês!

"O amor não vê com os olhos, vê com a mente; por isso é alado, é cego e tão patente."

Willian Shakespeare

Agradecimentos Especiais

Karina Sampaio Caiaffa

A Deus,

Obrigada meu querido amigo, por tudo que tens me proporcionado, por todos os sonhos alcançados e por me acompanhar em todos os momentos da minha vida, cuidado da minha maior riqueza, minha família, enquanto estava longe... O senhor é o meu guia e nada me faltará...

Agradeço-te imensamente.

AOS MEUS QUERIDOS AVÓS IRANY E OCTACÍLIO,

Sou eternamente grata pelos maiores ensinamentos que tive com vocês, acredito que boa parcela do que sou hoje devo aos conselhos e palavras sábias, por mais simples que fossem... Agradeço pelas orações diárias, e a Deus por ter me dado os avós mais doces do mundo, mesmo que a vida tenha sido tão dura com vocês! Agradeço por terem me ensinado o que é honestidade, sinceridade e fé. Tenho muito orgulho das minhas raízes no interior...

"Aprenda a viver dentro das suas possibilidades. Buscar uma vida de aparências, fora da sua realidade, só o levará para o abismo sem volta. Construa a sua vida aos poucos, lutando a cada dia e extraindo da vida o que ela tem de melhor: a Simplicidade." Chico Xavier

Ao meu irmão Marcos Paulo,

Obrigada meu pequeno grande homem, pelo carinho e torcida por minha felicidade....Desejo que um dia você se torne um homem de sucesso tanto na vida profissional quanto na vida sentimental... Te amo muito.

Ao meu querido Namorado Franci,

Encontrá-lo no dia do meu aniversário foi o maior presente que Deus poderia ter me dado... Agradeço todos os momentos que estou em sua companhia, sempre me alegrando e dando força pra continuar... Peço a Deus que ele possa nós unir para toda vida, para compensar todos esses anos longes um do outro... Que possamos construir muitas coisas juntos e que a felicidade nós acompanhe... Te amo muito!

"Fundamental é mesmo o amor, é impossível ser feliz sozinho." Tom Jobim

"E quando eu estiver triste... Simplesmente me abrace..." Skank

À Família de meu Namorado,

Jusemar, Antônio Lorenço, Wanderléia e Vanusa... Agradeço minha segunda família por todo apoio e carinho e por terem me recebido tão bem em sua casa, e principalmente por terem criado o grande amor da minha vida com tanta dedicação e carinho, tornando-o um homem honesto e tão especial... Conhecendo vocês é fácil entender que pessoas boas e honestas existem. Obrigada por tudo! Adoro todos vocês!

À minha querida Orientadora, Amiga e 2ª Mãe

Profa. Dra. Cristiane Duque,

Não sei nem por onde começar... Com os olhos cheios de lágrimas eu começo a escrever sobre essa pessoa, que me trouxe tantas alegrias, que se tornou um exemplo tão grande

para mim... Agradeço do fundo do meu coração por sempre estar presente em minha vida, escutando minhas angústias, minhas tristezas e principalmente dividindo o melhor que temos... Nossa amizade. Obrigada por todo conhecimento que me transmitiu. Se hoje me dedico de coração à pesquisa, devo isso a você.

Agradeço por sempre se preocupar comigo, pela compreensão, conversas e por todos os ensinamentos e momentos que passamos juntas.

Existem pessoas na vida da gente que entram para nunca mais sair... A senhora para mim é um exemplo de Honestidade, Moral, Mãe, Amiga, Professora dedicada, Filha querida, Esposa perfeita, entre outras mil qualidades... Eu costumo dizer que se eu me tornar a metade da metade do que a senhora representa... Já agradecerei a Deus por isso. Amo muito a senhora.

"Ninguém cruza nosso caminho por acaso
e nós não entramos na vida de ninguém sem nenhuma razão."

"Embora ninguém possa voltar atrás e fazer um novo começo,
qualquer um pode começar agora e fazer um novo fim." Chico Xavier

À querida Família Duque Talora

Luiz Fernando, Doraci, Sônia, Edenizia e ao menininho que conquistou meu coração, Felipe. Agradeço tudo que fizeram por mim... Pelo acolhimento dentro de suas casas e por todo carinho que sempre tiveram comigo... Sem vocês tudo teria sido muito mais difícil, principalmente pela distância da minha família de sangue, pois hoje posso dizer que ganhei uma nova família... Vocês significam muito para mim... Ensinaram-me muitas coisas boas e sou encantada pelo amor que existe dentro dessa família.

Às queridas Famílias Sampaio e Caiaffa

Ao apoio dado, carinho e torcida para que tudo desse certo nos meus estudos.
Por tudo o que já fizeram por mim, minha eterna gratidão. Minha admiração por vocês é imensa, assim como a alegria de tê-los sempre por perto. Obrigado por existirem em minha vida. Amo vocês.

À minha querida Mãe e querido Pai

Tudo que sou hoje devo a vocês, agradeço por terem me criado com dedicação e de ajudar a conquistar muita coisa na minha vida. Vocês foram essenciais na minha formação. Amo vocês!!
Obrigado por me ensinarem que caráter é aquilo que você é quando ninguém está olhando...

Ao meu querido Pai (Tio Paulão)

Por todo amor que me oferece, por tudo que me ensina e pelo carinho que sempre me trata. Hoje te agradeço por tudo que o senhor me ajudou, nos momentos mais difíceis era com você que eu podia contar... Dando-me força para não desistir... O amor que sinto por você é tão grande... Deus não poderia ter me presenteado melhor. Muitas coisas bonitas não podem ser vistas ou tocadas, elas são sentidas dentro do coração. O que o senhor faz por mim é uma delas.
Te amo muito!

Às minhas grandes Amigas Marcelle, Maria Luiza e Márjully

Agradeço minhas amigas por todo apoio, carinho e amizade... Nos momentos que me sentia triste, lembrava-me que vocês estavam comigo. Foram muitas conversas e

risadas, e que me alegravam mesmo nos momentos de dificuldade... Agradeço o companheirismo e a tudo que fizeram por mim.
Seremos eternamente amigas!

"Depois de um tempo você aprende que verdadeiras amizades continuam a crescer mesmo a longas distâncias".

Willian Shakespeare

Às minhas Amigas Maria Luiza, Loiane, Paula, Kelly, Natália Colombo e Jackeline

Agradeço por me ajudarei no laboratório e na vida, com cada uma de vocês pude aprender coisas que vou levar pra vida toda... Obrigada pela amizade e carinho!

Ao meu grande Amigo José Antônio

Pelo companheirismo, amizade e brincadeiras. Admiro muito sua força de vontade de vencer e agradeço nossos almoços sempre muito divertidos. Te adoro, meu amigo!

Aos companheiros de Laboratório Vanessa, Remberto, Juliana e Lucas

A ajuda de vocês em alguns momentos no laboratório
foram de grande importância, Obrigada!

Às alunas e alunos de Mestrado, Doutorado, Pós-Doutorado e Iniciação Científica

Aos meus amigos que sempre me acompanham na Odontopediatria: Douglas, Renan, Laís Areas, Kevin, Mariana, Thayse, Valéria, Dani Oliveira, Dani Camara, Jackeline, Jaqueline, Tatiana, Giovanna, Larissa, Sâmia, Lili, Gabriela, Guilherme, Carla, Carolina Lodi, Jesse, Lucas, Luis, Laís Ribas e Dinah.

Aos amigos da Endodontia: À minha querida amiga Francine, mesmo em pouco tempo de convivência juntas, sei reconhecer a pessoa maravilhosa que você é. Ao Carlos, Diego, Gabriele, Índia, Renata, Marcelo, Mariane, Luciana, Chris Men e Guilherme.

A amizade e colaboração de cada um foram essenciais para a conclusão desta pesquisa. Admiro todos vocês!

Aos docentes da Disciplina de Endodontia da Faculdade de Odontologia de Araçatuba, UNESP

Prof. Dr. Roberto Holland, Prof. Dr. Mauro Juvenal Nery, Prof. Dr. José Arlindo Otoboni Filho, Prof. Dr. Elói Dezan Júnior, Prof. Dr. João Eduardo Gomes Filho; Prof. Dr. Luciano Tavares Ângelo Cintra, Prof. Dr. Rogerio Castilho Jacinto e Prof. Dr. Gustavo Sivieri Araújo.

Por todos os ensinamentos a mim transmitidos, contribuindo na minha formação profissional.

Aos docentes da Disciplina de Odontopediatria da Faculdade de Odontologia de Araçatuba, UNESP

Prof. Dr. Alberto Carlos B. Delbem, Prof. Dr. Célio Percinoto, Prof. Dr. Robson Federico Cunha, Prof. Dr. Juliano Pelim Pessan e Profª Dra. Sandra M. H. C. Ávila de Aguiar, pela agradável convivência, acolhimento no departamento e conhecimentos transmitidos. Muito obrigada!

Aos professores colaboradores Prof. Dr. Eduardo M. Cilli, Profa. Dra. Denise M. Palomari Spolidorio e Prof. Dr. Carlos Alberto de Souza Costa

Obrigada por toda confiança gentilmente cedida ao trabalho e por toda ajuda que foi de fundamental importância para o andamento e finalização do trabalho. Muito obrigada!

Aos alunos Colaboradores do trabalho: Norival Alves Santos-Filho, Loiane Massunari, Marcelle Danelon, Telma Blanca Lombardo Bedran, Fernanda Basso, Gabriel Abuna e Lívia.

A ajuda de vocês foi de fundamental importância para a realização deste trabalho, agradeço de coração a paciência que vocês tiveram em me ensinar. Aprendi muito na companhia de vocês. Muito Obrigada!

Aos queridos Técnicos dos laboratórios da UNESP - Araçatuba, Araraquara e UNICAMP – Piracicaba: Ricardo, Juliana Pirola, Aline Zenatti, Flávia e Liliane.

Obrigada por toda ajuda e ensinamentos, aprendi muito com vocês. Todos que ajudaram, foram peças importantíssimas deste trabalho. Muito obrigada!!

"Nosso carinho e gratidão, a vocês, que além de transmitirem seus conhecimentos e suas experiências, souberam apoiar-nos em nossas dificuldades."

Aos funcionários do Departamento de Odontopeiatria

Ricardo, Mário e Luisinho. Por manterem a organização no laboratório; Por toda a ajuda e dedicação. Muito Obrigada!

Aos funcionários do Departamento de Endodontia

Nelci, Claúdia, Elaine, Peterson e Grazi. Mesmo nos poucos momentos que passei com vocês, agradeço a ajuda e sei o quanto profissionais dedicados são.

À Banca do Exame Geral de Qualificação e à Banca de Defesa

Muito obrigada por toda contribuição no trabalho e pelos conhecimentos trocados.

À Faculdade de Odontologia de Araçatuba, na pessoa dos professores Drª Ana Maria Pires Soubhia, digníssima Diretora e Dr. Wilson Roberto Poi, digníssimo Vice-Diretor.

Ao Curso de Pós-Graduação em Ciência Odontológica da Faculdade de Odontologia de Araçatuba-UNESP, na pessoa do coordenador Prof. Dr. Luciano Tavares Ângelo Cintra.

Aos funcionários da biblioteca da Faculdade de Odontologia de Araçatuba da UNESP, Ana Cláudia, Luzia, Ivone, Cláudio, Maria Cláudia, Luiz, Denise e Izamar pela atenção e disponibilidade com que nos recebem.

À Valéria, Cristiane e Lilian da Seção de Pós-Graduação da Faculdade de Odontologia de Araçatuba-UNESP, pelo profissionalismo e atenção sempre carinhosa.

Ao Frigorífico Friboi, que permitiram a coleta dos dentes bovinos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (Processo: 134551/2013-3), ao auxílio PROPe UNESP (Processo:0403/001/14) e à Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo: 2013/24606-5) pela concessão de recursos que possibilitou a realização deste Curso de Mestrado.

A todos que, de alguma forma contribuíram para a elaboração e conclusão deste trabalho...

Minha eterna gratidão...

Karina Sampaio Caiaffa

A arte de ser feliz

Houve um tempo em que minha janela se abria
sobre uma cidade que parecia ser feita de giz.
Perto da janela havia um pequeno jardim quase seco.
Era uma época de estiagem, de terra esfarelada,
e o jardim parecia morto.
Mas todas as manhãs vinha um pobre com um balde,
e, em silêncio, ia atirando com a mão umas gotas de água sobre as plantas.
Não era uma rega: era uma espécie de aspersão ritual, para que o jardim não morresse.
E eu olhava para as plantas, para o homem, para as gotas de água que caíam de seus dedos
magros e meu coração ficava completamente feliz.
Às vezes abro a janela e encontro o jasmineiro em flor.
Outras vezes encontro nuvens espessas.
Avisto crianças que vão para a escola.
Pardais que pulam pelo muro.
Gatos que abrem e fecham os olhos, sonhando com pardais.
Borboletas brancas, duas a duas, como refletidas no espelho do ar.
Marimbondos que sempre me parecem personagens de Lope de Vega.
Ás vezes, um galo canta.
Às vezes, um avião passa.
Tudo está certo, no seu lugar, cumprindo o seu destino.
E eu me sinto completamente feliz.
Mas, quando falo dessas pequenas felicidades certas,
que estão diante de cada janela, uns dizem que essas coisas não existem,
outros que só existem diante das minhas janelas, e outros,
finalmente, que é preciso aprender a olhar, para poder vê-las assim.
Cecília Meireles

Epígrafe

Karina Sampaio Caiaffa

Resumo Geral

Caiaffa KS. Efeito citotóxico e antimicrobiano de análogos de peptídeos catiônicos e sua influência na expressão de marcadores fenotípicos e genotípicos de mineralização dentinária**.** [Dissertação]. Araçatuba: Universidade Estadual Paulista; 2015.

Resumo Geral

Os objetivos do estudo foram avaliar os efeitos citotóxico e antimicrobiano de análogos de peptídeos catiônicos e sua influência na expressão de marcadores fenotípicos e genotípicos de mineralização dentinária**.** Fibroblastos da linhagem L929 foram expostas a diluições seriadas dos peptídeos LL-37 e análogos hBD-3-1C^{V} e KR-12-a5 e a viabilidade das células foi avaliada por ensaios de metil tiazol tetrazólio (MTT). Concentração inibitória mínima (CIM) e concentração letal mínima (CLM) para os peptídeos e controles (clorexidina – CHX) foram determinadas contra *Streptococcus mutans, Actinomyces israelii, Enterococcus faecalis, Candida albicans, Fusobacterium nucleatum* e *Porphyromonas gingivalis*, pelo método de microdiluição, após 4 e 24 horas. Biofilmes de *E. faecalis* e *F. nucleatum* foram formados sobre blocos de dentina radicular bovina e expostos a 5X e 10X MLC do peptídeo com melhor atividade antimicrobiana e CHX e analisadas por contagem de unidades formadoras de colônias/ml (UFC/ml) e por Microscopia de Varredura Confocal à Laser (CLSM). Células semelhantes à odontoblastos da linhagem MDPC-23 foram expostas a diluições seriadas de LL-37, hBD-3-1C^{V}, KR-12-A5 por 24 horas, seguidas de trocas de meio osteogênico por 7 dias e avaliada a viabilidade celular, produção de proteína total (TP), atividade da fosfatase alcalina (ALP) e deposição de nódulos mineralizados. A expressão de genes de marcadores de mineralização (sialofosfoproteína dentinária - DSPP e fosfoproteína de matriz dentinária - DMP-1) foi realizada por PCR quantitativo, após 24 h de exposição aos peptídeos e de incubação durante 14 dias em meio osteogênico. LL-37/hBD-3-1C^{V} e KR-12-a5 afetaram o metabolismo dos fibroblastos em concentrações acima de 500 e 250 µg/ml, respectivamente. KR-12-a5 teve os melhores valores de CIM/CLM contra todos os microrganismos e ambos os tempos de exposição. Os crescimentos de *E. faecalis* e *C. albicans* foram afetados apenas por KR-12-a5 e CHX. hBD-3-1C^{V} e LL-37 tiveram efeito inibitório semelhante contra *S. mutans* e *A. israelii*. KR-12-a5 reduziu o crescimento de *E. faecalis* em ensaios de biofilme, com resultados semelhantes à CHX. KR-12-a5 eliminou

100% do biofilme de *F. nucleatum*. Considerando a análise de CLSM, KR-12-a5 e CHX reduziram significativamente a viabilidade de *E. faecalis* dentro túbulos dentinários. LL-37 e hBD-3-1C^{V} afetaram minimamente o crescimento das células odontoblastóides em concentrações abaixo de 62,5 µg/ml. KR-12-a5 foi menos citotóxico abaixo de 31,25µg/ml. A produção de TP foi semelhante para todos os grupos em comparação ao grupo controle, exceto para hBD-3-1C^{V} (15,62 µg/ml). LL-37 (62,5 µg/ml) induziu maior atividade de ALP quando comparado com o controle e os outros grupos. LL-37 e hBD-3-1C^{V} à 62,5 µg/ml e KR-12-a5 à 31,25 µg/ml estimularam maior deposição de nódulos mineralizados. Considerando a expressão de DSPP e DMP-1, não foram observadas diferenças estatísticas entre os grupos ou quando eles foram comparados com o controle. Conclui-se que KR-12-a5 teve atividade antimicrobiana superior aos outros peptídeos e mostrou atividade antibiofilme similar à CHX, causando mínima toxicidade aos fibroblastos. Além disso, semelhante ao que ocorreu aos demais peptídeos testados, porém em concentração menor e minimamente citotóxica, KR12-a5 também estimulou a deposição inicial de nódulos mineralizados pelas células semelhantes a odontoblastos. Nenhum peptídeo afetou a expressão dos genes DSPP e DMP-1, envolvidos na mineralização ativa da dentina.

Palavras-chave: Peptídeos Catiônicos Antimicrobianos, Biofilmes e Expressão Gênica.

General Abstract

Karina Sampaio Caiaffa

Caiaffa KS. Cytotoxic and antimicrobial effects of analogues of cationic peptides and their influence in the expression of phenotypic and genotypic markers of dentin mineralization. [Dissertação]. Araçatuba: Universidade Estadual Paulista; 2015.

General Abstract

The objectives of the study were to evaluate the cytotoxic and antimicrobial effects of analogues of cationic peptides and their influence in the expression of phenotypic and genotypic markers of dentin mineralization. L929 fibroblast cells were exposed to serial dilutions of peptides LL-37, hBD-3-1C^{V} and KR-12-a5 and cell metabolism was evaluated by methyl thiazol tetrazolium (MTT) assays. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of peptides and controls (chlorhexidine – CHX) were determined for *Streptococcus mutans, Actinomyces israelii, Enterococcus faecalis, Candida albicans, Fusobacterium nucleatum* and *Porphyromonas gingivalis*, by microdilution method, after 4 and 24h. *E. faecalis* and *F. nucleatum* biofilms were formed in blocks of bovine root dentin and exposed to 5X and 10X MLC of the peptide with the best antimicrobial activity and CHX and analyzed by colonies forming units/ml (CFU/ml) counts and by Confocal Laser Scanning Microscopy (CLSM). MDPC-23 odontoblast-like cells were exposed to serial dilutions of peptides LL-37, hBD-3-1C^{V} and KR-12-a5 for 24 hours, followed by changes of osteogenic medium for 7 days and evaluated cell viability, total protein (TP) production, alkaline phosphatase (ALP) activity and mineralized nodule deposition. The gene expression of mineralization markers (Dentin Sialophosphoprotein - DSPP and Dentin matrix phosphoprotein 1 - DMP-1) was performed by quantitative PCR, after 24h of peptide exposure and incubation for 14 days in osteogenic medium. LL-37 and hBD-3-1C^{V} affected cell metabolism at concentrations above 500µg/ml and KR-12-a5 above 250µg/ml. KR-12-a5 had the best MIC/MLC values against all microorganisms and both times of exposure. *E. faecalis* and *C. albicans* growth was affected only for KR-12-a5 and CHX. hBD-3-1C^{V} and LL-37 had similar inhibitory effect against *S. mutans* and *A. israelii.* KR-12-a5 reduced *E. faecalis* growth in biofilm assays, with similar results to CHX. KR-12-a5 killed 100% of *F. nucleatum* biofilm. Considering CLSM analysis, KR-12-a5 and CHX significantly reduced *E. faecalis* viability inside dentin tubules. LL-37 and hBD-3-1C^{V} affected minimally

odontoblastic-like cells growth at the concentrations below 62.5 μg/ml. KR-12-a5 was less cytotoxic below 31.25 μg/ml. TP production was similar for all groups compared with the control group, except by hBD-3-1C^{V} (15.62 μg/ml). LL-37 (62.5 μg/ml) induced higher ALP activity than control and another groups. LL-37 and hBD-3-1C^{V}, at 62.5 μg/ml and KR-12-a5 at 31.25 μg/ml stimulated the highest deposition of mineralized nodules. Considering DSPP and DMP-1 expression, no statistical differences were observed among the groups or when they were compared with the control. This study concluded that KR-12-a5 had superior antimicrobial activity compared to another peptides and showed anti-biofilm activity similar to CHX, causing minimal toxicity to fibroblast cells. Besides, KR-12-a5 also stimulated initial mineralized nodules deposition, similar to another peptides, but in lower and minimally cytotoxic concentration. None peptides influenced the expression of DSPP and DMP-1, genes involved in active dentin mineralization.

Keywords: Antimicrobial Cationic Peptides, Biofilms and Gene Expression

Lista de Figuras

Lista de Figuras

Capítulo 1

Capítulo 2

Lista de Tabelas

Lista de Tabelas

Capítulo 1

Capítulo 2

Lista de Abreviaturas

Lista de Abreviaturas

µg/ml: Microgram per milliliter; Micrograma por mililitro

ALP: Alkaline phosphatase activity ; Atividade de fosfatase alcalina

AMPs: Antimicrobial peptides; Peptídeos catiônicos antimicrobianos

APT: Amphotericin; Amfotericina

ATCC: de American Type Culture Collection; Coleção Americana de tipos de cultura

BHI: Brain Heart Infusion Agar; *Agar* Infusão de Cérebro e Coração

CFU/ml - UFC/ml: Colony forming unit per milliliter; Unidade formadora de colônia por mililitro

CFU: Colony-forming units; Unidades formadoras de colônia

CHX: Chlorhexidine digluconate; Clorexidina

CLM/CLM: Minimal lethal concentration; Concentração letal mínima

CLSM: Confocal Laser scanning Microscopy, Microscopia de varredura à laser

cm: Centimeters; Centímetros

DMP-1: Dentine matrix phosphoprotein 1; Fosfoproteína de matriz dentinária 1

DNA: Deoxyribonucleic acid; Ácido desoxirribonucleico

DSPP: Dentine sialophosphoprotein; Sialofosfoproteina dentinária

GAPDH: Glyceraldehyde-3-phosphotedehydrogenase

h: Hours; Horas

hBD: : Human β-defensin; β-defensina humana

hBD-1: Human β-defensin 1; β-defensina 1 humana

hBD-2: Human β-defensin 2; β-defensina 2 humana

hBD-3: Human β-defensin 3; β-defensina 3 humana

hBD-3-1C^{V}: Human β-defensin 3 analogue; Análogo da β-defensina Humana 3

hNP1-4: Human neutrophil defensins (α-defensin); α-defensina

KR-12-a5: Human cathelicidin LL-37 analogue; Análogo da catelicidina humana LL-37

LL-37: Human cathelicidin LL-37; Catelicidina humana LL-37

MIC/CIM: Minimal inhibitory concentration; Concentração inibitória Mínima

MTT: Methyl thiazol tetrazolium; Metil tiazol tetrazólio

PCR: Polymerase Chain Reaction; Reação em cadeia da polimerase

qPCR: Quantitative Real-Time PCR; PCR quantitativo em tempo real

RNA: Ribonucleic acid; Ácido ribonucleico

TP:Total protein production; Produção de proteína total

Sumário

Sumário

Introdução Geral

Introdução Geral

O tratamento de dentes permanentes jovens que sofreram danos irreversíveis devido à infecção ou trauma local antes do fechamento fisiológico normal do ápice radicular representa um verdadeiro desafio clínico. Após a erupção completa do dente, ainda são necessários 3 a 4 anos para o desenvolvimento completo dos canais e o fechamento dos ápices radiculares. Durante este período, se ocorrer algum tipo de trauma ou infecção, esses processos são interrompidos e o fechamento natural do ápice não acontece. As paredes dentinárias finas divergentes ou paralelas do dente imaturo dificultam a desinfecção e a execução dos procedimentos endodônticos convencionais, comprometendo o resultado em longo prazo (Rafter, 2005; Wang et al., 2010; Iglesias-Linares et al., 2013).

O material mais indicado para o tratamento de dentes permanentes jovens com comprometimento pulpar/periapical é o hidróxido de cálcio (HC). Diversos trabalhos têm mostrado que este material apresenta biocompatibilidade e atividade antimicrobiana, além de estimular a apicificação nestes dentes (Sheehy e Roberts, 1997; Shabahang e Torabinejad, 2000; Rafter, 2005; Parirokh e Torabinejad, 2010; da Silva et al., 2010). Entretanto, atualmente há uma corrente de pesquisadores estudando materiais biológicos que possam preservar a bainha epitelial de Hertwig e estimular a diferenciação de células da papila apical em odontoblastos (Thibodeaud et al., 2007; Jung et al., 2008; Ding et al., 2009; Taneja et al., 2010, Ishizaka et al., 2012; Iglesias-Linares et al., 2013). Esse tecido é indubitavelmente removido por meio dos tratamentos químico-mecânico tradicionais (Banchs e Trope, 2004, Iglesias-Linares et al., 2013). Além disso, embora o HC seja reconhecidamente considerado o medicamento de escolha, ainda não existe um material ideal para a realização do tratamento endodôntico de dentes permanentes jovens, levando ao estudo de novas alternativas biológicas.

As doenças endodônticas ocorrem como resultado da invasão da microbiota oral autógena na dentina e em seguida, no sistema de canais radiculares e tecidos perirradiculares. Assim, o primeiro passo da terapia endodôntica deve ser a eliminação dos microrganismos e a prevenção de reinfecção nos canais radiculares (Nair, 2004). Entretanto, 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). Segundo Lopes e Siqueira (1999), idealmente a medicação intracanal deve promover a eliminação e impedir a proliferação de microrganismos remanescentes, atuar como barreira físico-química contra a infecção ou a reinfecção por microrganismos, reduzir a inflamação perirradicular, neutralizar produtos tóxicos, controlar a reabsorção dentinária inflamatória externa, além de estimular a reparação por tecido mineralizado.

Amostras dos canais radiculares têm sido obtidas no momento do tratamento para acessar a eficácia dos protocolos clínicos e/ou para avaliar as condições bacteriológicas do canal após o preparo químico-mecânico. Diversos autores têm detectado uma média de 1 a 5 espécies bacterianas após preparo químico-mecânico, seguido ou não de medicação intracanal, atingindo em torno de 10^2 a 10^5 células/canal (Byström e Sundqvist, 1985; Sjögren et al, 1997; Vianna et al., 2006b; 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 108 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 microbiana, 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 frequentemente presentes nestes casos, incluindo estreptococos (*S. mitis, S. gordonii, S. anginosus, S. sanguinis, S. oralis*), *P. micra*, *Actinomyces* especies (*A. israelii* e *A. odontolyticus*), *Propionibacterium* especies (*P. acnes* e *P. propionicum*), *P. alactolyticus*, lactobacilos (*L. paracasei, L. acidophilus*), *Enterococcus faecalis*, entre outros (Siqueira e Rôças, 2009).

Mesmo eliminando as bactérias, diversas toxinas são liberadas no canal radicular durante a infecção e quando em contato com as células do hospedeiro levam a resposta inflamatória. Entre esses fatores de virulência, estão o ácido lipoteicóico (LTA) e o

lipopolissacarídeo (LPS). O LTA está localizado na parede celular de bactérias gram-positivas e quando em contato com leucócitos estimula a liberação de mediadores inflamatórios (Han et al., 2003; Baik et al., 2008). Também tem mostrado alta afinidade a hidroxiapatita, um aspecto que poderia facilitar a sua penetração nos túbulos dentinários (Ciardi et al., 1977). LPS é o maior constituinte da membrana externa das bactérias gram-negativas. Embora o mecanismo exato do LPS não esteja completamente elucidado, sabe-se que age ativando fagócitos mononucleares (monócitos e macrófagos), que consequentemente aumentam sua atividade fagocítica e a secreção de citocinas pró-inflamatórias, tais como, fator de necrose tumoral (TNF-α), interleucinas (IL-1β, IL-6, entre outras), prostaglandinas (PGE2). Sabe-se que a resposta inflamatória é uma aliada ao processo de reparo, entretanto, a secreção descontrolada dessas citocinas induz a expressão de RANKL (ligante do receptor de NF-κβ), um crítico fator de diferenciação dos osteoblastos em clastos, podendo levar à destruição tecidual (Raetz e Whitfield, 2002; Wash et al., 2006). A neutralização dessas toxinas bacterianas é uma importante característica para a medicação intracanal, pois somente o preparo químico-mecânico (com hipoclorito de sódio, por exemplo) não tem se mostrado efetivo em eliminar LPS (De Oliveira et al., 2007) ou LTA bacteriano (Hartke et al., 1998).

Os peptídeos catiônicos antimicrobianos (PCAM) estão sendo estudados como alternativas aos antibióticos tradicionais na eliminação de microrganismos em diversos campos da Medicina e, atualmente, na Odontologia (Hözl et al., 2008; Gorr e Abdolhosseini, 2011). Eles representam uma família de peptídeos encontrados na cavidade bucal e em outros locais do organismo. São formados a partir do fracionamento de proteínas e da obtenção de fragmentos funcionais. Estes apresentam ação contra uma ampla variedade de bactérias, fungos e vírus envelopados e, além disso, promovem modulação da resposta imune do hospedeiro, mantendo a microbiota normal em estado estável em diferentes nichos, como a pele, os intestinos e a cavidade bucal (Mccormick e Weinberg, 2010; Wiesner e Vilcinskas, 2010). Os principais PCAM presentes na saliva e também no fluido crevicular são as defensinas e catelicidinas. As defensinas são peptídeos pequenos, de 15 a 45 aminoácidos, que dependendo do padrão de pareamento de seus resíduos de cisteína, são subdivididas em duas principais subfamílias: α e β-defensinas. Foram identificadas seis α-defensinas em humanos, sendo que quatro são produzidas pelos neutrófilos e denominadas de peptídeo neutrofílico

humano (HNP-1 a 4) e as outras duas são produzidas por células de Paneth nas criptas intestinais. As β-defensinas (hBDs)são produzidas por células epiteliais de diversos órgãos como olhos, pele, pulmão, rim, pâncreas, mucosa nasal e oral e embora tenham sido encontradas quase 40 regiões gênicas potenciais para hBDs, as mais bem caracterizadas são hBD 1 a 4. As α e β-defensinas apresentam função imunomoduladora, modificando a migração e maturação celular, induzindo citocinas e a liberação de histamina e prostaglandina A2 de mastócitos (Abiko, 2003; Mccormick e Weinberg, 2010; Wiesner e Vilcinskas, 2010).

O peptídeo catiônico humano (hCAP-18) é a única catelicidina identificada em seres humanos isolada primeiramente em grânulos de neutrófilos. hCAP-18 é produzida também por células epiteliais do pulmão, intestino, cavidade bucal e trato urogenital, sendo encontrada no plasma seminal e plasma sanguíneo. Após a secreção, ocorre a quebra de hCAP-18 pela ação de proteases em pequenos fragmentos de peptídeos RK-31 e KS-30 e em um peptídeo ativo de cadeia longa LL-37, todos com ação antimicrobiana. Esse último fragmento do peptídeo hCAP-18, o LL-37, é um modulador multifuncional da imunidade inata, envolvendo a função antibacteriana, estímulo de angiogênese, cicatrização cutânea e quimiotaxia de células inflamatórias e do sistema imune. LL-37 atenua a produção de citocinas induzidas por LPS e a expressão de quimiocinas por fibroblastos (Jönsson e Nilsson, 2012). A ação antimicrobiana de LL-37 está relacionada à formação de poros na membrana das bactérias e a lise celular, entretanto em altas concentrações (>13μM) pode se tóxica para as células eucarióticas (Zhang et al., 2008; Mccormick e Weinberg, 2010).

Dentre os PCAMs, a hBD-3 e LL37 tem mostrado excelente ação antimicrobiana contra patógenos orais, incluindo bactérias gram-positivas e gram-negativas facultativas ou estritas, como *A. actinomycetemcomitans, P. gingivalis, P. intermedia, F. nucleatum, S. mutans, S. sobrinus, S. salivarius, S. sanguis, S. mitis e L. casei* (Ouhara et al., 2005) e fungos, como *C. albicans* (Harder et al., 2001; Wong et al., 2011). Além disso, esses PCAM tem mostrado ação antimicrobiana e atividade neutralizante de LPS de *P. intermedia* e *T. forsythia,* no caso do hBD-3 (Lee et al., 2010) e *A. actinomycetemcomitans, P. gingivalis, P. intermedia e F. nucleatum* para LL-37 (Suphasiriroj et al., 2013). Foi verificado também efeito antimicrobiano de hBD-3 sobre biofilme de *E. faecalis* (Lee et al., 2013a) e sobre biofilme multiespécie, contendo *A. naeslundii, Lactobacillus salivarius, S. mutans e E.*

faecalis (Lee et al., 2013b), além de ação neutralizante contra LTA de *E. faecalis* (Lee e Baek, 2012).

Devido ao alto custo para a obtenção dos PCAM, diversos estudos têm proposto a utilização de peptídeos sintéticos recombinantes, como rhBD-3 (Song et al., 2009) que obteve excelentes resultados não somente contra *S. aureus* ou *E. coli*, mas também para alguns microrganismos patogênicos encontrados em canais radiculares, como *S. mutans, A. naeslundii, E. faecalis e C. albicans*. Outra alternativa é a redução dos fragmentos ou modificação dos mesmos, buscando manter sua ação antimicrobiana e imunorregulatória. Taylor et al. (2008) mostraram que a alteração na sequência da hBD-3, substituindo 5 cisteínas por alaninas, mantendo somente uma cisteína sem ponte dissulfídica (HBD3-1Cv) ainda manteve sua ação antimicrobiana contra *P. aeruginosa* e *S. aureus* e ação quimiotática para células expressando receptor CCR-6, como monócitos e células T. Para LL-37, também foram propostas reduções no tamanho do fragmento, obtendo-se um menor fragmento com a mesma atividade antimicrobiana, denominado KR-12 (Wang, 2008). Modificações neste fragmento foram realizadas por Jacob et al. (2013) e foi verificado que alguns análogos de KR-12 apresentaram potente ação antimicrobiana contra cepas de *S. aureus* meticilina-resistentes e mantiveram a ação neutralizante contra LPS, sem causar toxicidade às células eucarióticas. Destes, o fragmento KR-12-a5 apresentou a maior atividade antiendotóxica, quase similar a do próprio LL-37.

Além da ação antimicrobiana e anti-inflamatória, é interessante que uma medicação seja capaz de estimular o tecido vital residual da bainha epitelial de Hertwig nos dentes imaturos e auxiliar na diferenciação das células em odontoblastos para que haja deposição de dentina e o fechamento natural do ápice radicular. Estudo *in vitro* mostrou que LL-37 ativa EGFR (receptor do fator de crescimento epitelial) e JNK (quinase N-terminal c-Jun), moléculas envolvidas na migração e proliferação celular em células pulpares humanas, demonstrando que esse PCAM pode contribuir para a regeneração tecidual (Kajiya et al., 2010). As beta-defensinas também tem mostrado estimular a diferenciação de odontoblastos, por meio do aumento na síntese de sialofosfoproteína dentinária (DSPP) e osteopontina (OPN), marcadores sintetizados por células derivadas da polpa dentária (Shiba et al., 2003). Atividade adicional estimulando a proliferação, a diferenciação e a biomineralização por células semelhantes a

osteoblastos também foi documentada para as beta-defensinas, além de aumentar a atividade da enzima fosfatase alcalina (ALP) e a expressão de marcadores osteogênicos como BMP-2, BMP-4, entre outros e das próprias beta-defensinas (Kraus et al., 2011). Monócitos tratados com LL-37 se diferenciaram em células capazes de formar osso, expressando proteínas intracelulares da linhagem tanto de osteblastos quanto de osteoclastos, como osteocalcina (OC), osteonectina (ON), sialoproteína óssea II (BSP II), osteopontina (OP), entre outros (Zhang e Shively, 2010). Kittaka et al. (2013) mostraram que LL-37 foi capaz de regular a angiogênese e induziu o recrutamento de células indiferenciadas para promover regeneração óssea em modelo de defeito calvariano em ratos.

Baseado na filosofia de utilizar materiais biocompatíveis com propriedades antimicrobianas, anti-inflamatórias e ainda que possam estimular a completa formação apical, que novos protocolos de tratamento estão surgindo para dentes permanentes jovens (Iglesias-Linares et al., 2013; Moreno-Hidalgo et al., 2013). Assim, este presente trabalho pretende avaliar os efeitos citotóxico e antimicrobiano de análogos de peptídeos catiônicos e sua influência na expressão de marcadores fenotípicos e genotípicos de mineralização dentinária**.**

*Referências da Introdução Geral em Anexo O

Capítulo 1

Cytoxicity and inhibitory activity of analogues of cationic peptides on planktonic growth and biofilm of microorganisms associated with endodontic infections

***The manuscript is according to the guide for authors of Journal of Endodontics (Anexo A).**

Karina Sampaio Caiaffa[1], Loiane Massunari[1], Marcelle Danelon[5], Gabriel Flores Abuna[2], Telma Blanca Lombardo Bedran[3], Norival Alves Santos-Filho[4], Alberto Carlos Botazzo Delbem[5], Eduardo Maffud Cilli[4], Denise Madalena Palomari Spolidorio[3], Cristiane Duque[5]*

[1] Department of Endodontics, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba,São Paulo, Brazil.

[2] Department of Restorative Dentistry, Piracicaba Dental School, Universidade Estadual de Campinas (UNICAMP), Piracicaba,São Paulo, Brazil.

[3] Department of Physiology and Pathology, Araraquara Dental School, Univ Estadual Paulista (UNESP),Araraquara,São Paulo, Brazil.

[4]Department of Biochemistry and Chemical Technology, Institute of Chemistry, UNESP, Univ Estadual Paulista, Araraquara, São Paulo, Brazil

[5] Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, São Paulo, Brazil.

***Corresponding author:**

Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)

Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil

Tel: (+55) 1836363315

E-mail: cristianeduque@yahoo.com.br, cduque@foa.unesp.br

The authors declare that have no conflicts of interest.

Abstract

Introduction: The objectives of the study were to evaluate the cytoxicity and inhibitory activity of analogues of cationic peptides on planktonic growth and biofilm of microorganisms associated with endodontic infections.

Methods: L929 fibroblast cells were exposed to serial dilutions of peptides LL-37, hBD-3-1C^{V} and KR-12-a5 and cell metabolism was evaluated by MTT assays. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of peptides and controls (Chlorhexidine – CHX) were determined for *Streptococcus mutans, Actinomyces israelii, Enterococcus faecalis, Candida albicans, Fusobacterium nucleatum* and *Porphyromonas gingivalis*, by microdilution method, after 4 and 24h. *E. faecalis* and *F. nucleatum* biofilms were exposed to 5X and 10X MLC of the best antimicrobial peptide and analyzed by CFU/ml counts and by Confocal Laser Scanning Microscopy (CLSM).

Results: LL-37 and hBD-3-1C^{V} affected cell metabolism at concentrations above 500μg/ml and KR-12-a5 above 250μg/ml. KR-12-a5 had the best MIC/MLC values against all microorganisms and both times of exposure. *E. faecalis* and *C. albicans* growth was affected only for KR-12-a5 and CHX. hBD-3-1C^{V} and LL-37 had similar inhibitory effect against *S. mutans* and *A. israelii.* KR-12-a5 reduced *E. faecalis* growth in biofilm assays, with similar results to CHX. KR-12-a5 and CHX killed 100% of *F. nucleatum* biofilm. Considering CLSM analysis, KR-12-a5 significantly reduced viable cells inside dentin tubules similar to CHX.

Conclusion: KR-12-a5 had superior inhibitory activity compared to another peptides and showed anti-biofilm activity similar to CHX, causing minimal toxicity to fibroblast cells. KR-12-a5 is a potential antimicrobial agent and could be used for endodontic application.

Keywords: Cationic Antimicrobial Peptides; Microbial Sensitivity Tests; Biofilms; Cell Culture, Endodontics.

Introduction

Endodontic diseases occur as a result of autogenous oral microbiota invasion inside root canals and periradicular tissues. Thus, the first step of the endodontic therapy should be the elimination of microorganisms and prevention of reinfection in root canals (1). However, due to the complex system of accessory and lateral canals and the persistence of some microbial species, the chemical-mechanical preparation does not allow the full disinfection of the root canal system, becoming necessary the use of intracanal medication (2, 3). Gram-negative bacteria, common members of primary infections, are generally eliminated from root canal systems, after chemical-mechanical treatment. Exceptions may include some anaerobic bacilli such as *Fusobacterium nucleatum*, *Prevotella* species and *Campylobacter rectus*, which are among the species found in samples obtained from root canal after instrumentation/medication. However, most studies have shown that Gram positive bacteria are the most often present in these conditions, including streptococci *(S. mitis, S. gordonii, S. anginosus, S. sanguinis, S. oralis*), *P. microns, Actinomyces* especies (*A. israelii* and *A. odontolyticus*), *Propionibacterium* especies (*P. acnes and P. propionicum*), *P. alactolyticus*, lactobacilli (*L. paracasei, L. acidophilus*), *Enterococcus faecalis* and others (4).

Alternatives to traditional antibiotics have been studied for the elimination of microorganisms in Dentistry (5). Cationic antimicrobial peptides (AMP) can be found in several organs and fluids of the human body including saliva and gingival crevicular fluid. Defensins and cathelicidins are of the most common and important AMP found in oral cavity. Defensins are small peptides (15 to 45 amino acids), subdivided into two main subfamilies: α and β-defensins, which differ in the spacing and the pairing of the cysteine residues. Six α-defensins have been identified in humans and four of them are produced by neutrophils, designated human neutrophil peptide (hNP-1 to 4) and commonly found in gingival crevicular fluid. The β-defensins (hBDs) are produced by epithelial cells in various organs, including oral mucosa and the well-characterized are hBD- 1, -2 and -3. The α and β-defensins have immunomodulatory function that modify cell migration and maturation inducing cytokines and the release of histamine and prostaglandin A2 of mast cells (6, 7). The only human cathelicidin known as human cationic peptide-18 (hCAP-18) is produced by epithelial cells of the lung, intestines, oral cavity and urogenital tract. The hCAP-18 is broken by the action of proteases on small peptides. One of them and the most important is the peptide fragment LL-37 which has

wide broad antimicrobial activity (8) and exerts the immunomodulatory effects on several immune and non-immune cells, including chemotatic effect of neutrophils and eosinophils (9), cyclooxygnesase-2 expression and prostaglandin E2 production in gingival fibroblasts (10). LL-37 also attenuates cytokine production induced by LPS and the expression of chemokines in fibroblasts (11).

Among AMPs, hBD-3 and LL-37 have shown excellent antimicrobial activity against oral pathogens, including gram-positive and gram-negative facultative or strict bacteria such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Streptococcus mutans, S. sobrinus, S. salivarius, S. sanguis,* and *S. mitis, Lactobacillus casei* (12) and fungi such as *Candida albicans* (13). Although cationic peptides have been pointed as a new class of antibiotics, long length of their amino acid chain or chemical linkages difficult their production as therapeutic agent. Short peptides or analogues based in original AMPs have been attracted attention because of the potentially lower cost of production and optimization of their antimicrobial and immunological properties (14, 15, 16). Taylor et al. (14) synthesized an analog of hBD-3, called hBD-3-1c^{V} with five of the six cysteines changed to alanines, leaving only the fifth cysteine on its position. This peptide analogue was equally chemotactic for cells expressing receptor CCR-6, including monocytes and T cells and maintained the inhibitory effect against *P. aeruginosa* and *S. aureus* compared to original hBD-3. Jacob et al. (16) designed a series of analogues of the smallest peptide of LL-37 with antimicrobial activity, called KR12. Among these analogues, KR-12-a5 exhibited potent antimicrobial against important Gram positive and Gram negative bacteria and the highest LPS-binding activity. The objective of the study was to evaluate the cytoxicity and inhibitory activity of analogues of cationic peptides on planktonic growth and biofilm of microorganisms associated with endodontic infections.

Material and methods

Peptides synthesis (Análise da síntese dos peptídeos em Anexo B)

The peptides used in this study and their amino acid sequence were: cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRI-TES) (17), KR-12-a5 (LL-37 analogue - KRIVKLILKWLR-NH2) (16) and hBD-3-1C^{V} (human β-defensin-3 analogue - GIINTLQKYYARVRGGRAAVLSALPKEEQIGKASTRGRKCARRKK) (14). All peptides were

synthesized at Institute of Chemistry, UNESP, Araraquara, SP, Brazil. Solid phase peptide synthesis was performed manually by the method previously described by Merrifield, (18), using Fmoc (9-fluorenylmethyloxycarbonyl) protocols on the Rink-amide resin. The Fmoc group was deprotected by 20% piperidine/dimethylformamide (DMF). Coupling was performed at a twofold excess over the amino component in the resin using diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt) in a solution of 50% (v/v) DCM (dichloromethane) and DMF. After 2 h of incubation, the coupling was assessed by the ninhydrin test (19). Cleavage from the resin and removal of the protecting groups from the amino acid residue side chains were performed simultaneously with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) for LL-37 and KR-12-a5 and 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% 1,2-ethanedithiol (EDT) and 1% triisopropylsilane (TIS) for hBD-3-1C^{v}, both for 2 h. After this procedure, the crude peptides were precipitated with anhydrous ethyl ether and separated from the non-soluble material by centrifugation of the peptides. Subsequently, the peptides were extracted with 0.045% (v/v) TFA/H_2O (solvent A) and lyophilized for further purification. Molecular masses of peptides were estimated by mass spectrometry, using positive ion-mode electrospray ionization (ESI) apparatus (Bruker, Germany) and were in agreement with corresponding calculated values. Purification of synthesized peptides was performed in a semi-preparative HPLC Beckman System Gold on a reverse phase C18 column (2.1 × 25 cm, Phenomenex, Torrance, CA, USA). Final purity levels of peptides were determined at least 95% on a Shimadzu chromatography equipped with an analytical C18 reverse phase column (0.46 × 25 cm, Kromasil, Bohus, Sweden).

Preparation of antimicrobial agents

Peptides were weighed in an analytical balance (OHAUS Adventurer, Parsippany, NY, USA) at the concentration of 4 mg/ml and dissolved in sterile deionized water, except by hBD-3-1C^{V}, which was dissolved in 0.045% (v/v) TFA/H_2O. Chlorhexidine digluconate (CHX) and amphotericin (APT) (both from Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile deionized water to a concentration of 20 mg/ml. All solutions were filtered with 0.2µm syringe filters (Kasvi, Curitiba, PR, Brazil).

Cytotoxicity assays (Protocolo Anexo H)

Fibroblast cells and growth conditions

Fibroblast cells from L-929 lines were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO2 and 95% air at 37°C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). Cell cultures were sub-cultured every 2 days until reach the formation of a monolayer with an adequate number of cells for subsequent assays (20).

Analysis of cell metabolism

Fibroblast cells were submitted to trypsin treatment (TrypLETM Express, Life Technologies Inc.) for 5 min at 37°C. Proteases were then inactivated by adding 0.3 mg/ml of trypsin inhibitor, and the cells were harvested by centrifugation (500xg for 5 min), suspended in fresh medium, seeded in a 96-well microplate (200µl/well, $7x10^5$ cells/ml) and incubated 24h at 37°C in a 5% CO2 atmosphere to allow cell adhesion before stimulation. After that, cells were then stimulated with peptides and the controls CHX and APT at the concentration of 0.24 to 2000 µg/ml for 24 h in the same conditions. The colorimetric methyl tetrazolium (MTT) assay was used to evaluate cell metabolism by the cytochemical demonstration of succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells.

For the MTT assay, after antimicrobial agents stimulation, the culture medium was aspirated and 90μL of fresh DMEM (without fetal serum bovine) and 10μL of MTT solution (5 mg/ml in phosphate-buffered saline) (Sigma-Aldrich) were added to each well and incubated at 37°C for 4 h. Thereafter, the culture medium with MTT solution was aspirated, and the formazan crystals, resulting from the cleavage of the MTT salt ring by the SDH enzyme, were solubilized with acidified isopropanol solution (0.04 N HCl). An ELISA plate reader (BIO-RAD, model 3550-UV, Hercules, CA, USA) was used to assess cell viability proportional to the absorbance value determined by spectrophotometry at a 570 nm wavelength. The values obtained from the 2 aliquots were averaged to provide a single value for each well. The means were calculated for the groups and transformed into percentages of cell viability in relation to negative control (DMEM), which was defined as having 100% cell metabolism (20).

Antimicrobial activity

Microbial strains and growth conditions

In order to evaluate the antimicrobial activity of the peptides, the following standard strains were used: facultative anaerobic bacteria - *Enterococcus faecalis* (ATCC 51299), *Streptococcus mutans* (ATCC 25175), *Actinomyces israelii* (ATCC 12102), and strict anaerobic bacteria - *Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (NCTC 11326) and aerobic yeast *Candida albicans* (ATCC 26790). All strains were kindly provided by Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil). The purity of the strains was confirmed by the Gram's method (Protocolo Anexo C). Microbial suspensions were prepared from culture 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 24 h in a 5% CO_2 atmosphere (Incubator Ultra Safe, HF212-UV). *Candida albicans* were cultured in Sabouraud Dextrose Agar (SDA) (Difco Laboratories) containing 40 mg/ml chloramphenicol (Sigma-Aldrich) at 37°C in aerobic conditions. *P. gingivalis* and *F. nucleatum* were grown in Blood Agar - BHI Agar (Difco Laboratories) containing 5 mg/ml hemin, 10mg/ml menadione, yeast extract powder (YE, Difco Laboratories) and 5% defibrinated sheep blood at 37° C in anaerobic chamber (10% H_2, 10% CO_2 and 80% N_2) (Don Whitley Scientific MG500, West Yorkshire, UK). For subsequent microbiological assays, standard optical density was established for each microorganism: 0.5 for facultative anaerobic bacteria and *F. nucleatum* (approximately 1-5x10^8 CFU/ml), 0.2 for *P. gingivalis* (approximately 1-5x10^3 CFU/ml) and 0.3 for *Candida albicans* (approximately 1-5x10^6 CFU/ml) at 550-600nm. The absorbance was measured in 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) (Protocolo Anexo D)

Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were obtained by the microdilution method using 96-well microtiter plates, based on the criteria of the National Committee for Clinical Laboratory Standards

(NCCL) (21) for yeast and the National Committee for Clinical Laboratory Standards (NCCL) (22) for bacteria and according to Mor et al. (23) with 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 *Candida albicans* were cultured aerobically in RPMI-1640 medium (Sigma-Aldrich) under agitation at 135 rpm and 35°C. *F. nucleatum* and *P. gingivalis* were grown in BHI broth containing 5 mg/ml hemin, 10mg/ml menadione and yeast extract (YE) in anaerobic chamber for 48 and 72h, respectively. Microbial cultures were grown until reach the standard optical density - OD (described on item "Microbial strains and growth conditions") and harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at 3000xg, the supernatant was discarded and the pellet re-suspended in 2x concentrated Mueller-Hinton broth (Difco Laboratories), 2x concentrated RPMI-1640 medium (Sigma-Aldrich) for *C. albicans* and 2x concentrated BHI broth containing hemin, menadione and YE for anaerobic bacteria. The final concentration of bacterial suspension inside the wells was 1-5x10^5CFU/ml and 1-5x10^3CFU/ml for *Candida albicans*. Chlorhexidine digluconate (CHX) and amphotericin B (APT) were used as positive controls for bacteria and *C. albicans*, respectively and cultures without antimicrobials agents as negative control. All antimicrobial agents, except hBD-3-1C^V, which was diluted in 0.045% TFA solution, were serially diluted in sterile deionized water, in order to obtain the concentrations ranging from 0.24µg/ml to 4000µg/ml. The microbial suspensions were inoculated in each well containing the peptides previously diluted. The microplates were incubated at 37°C for 4 and 24 h for all microorganisms, except *F. nucleatum* and *P. gingivalis* that were incubated for 48 and 72h, respectively. Afterwards, 15µl of 0.01% resazurin (R7017 Sigma-Aldrich) was applied in each well and incubated for 4 h 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 facultative anaerobic bacteria, SDA for *C. albicans* and in Blood Agar for anaerobic bacteria for 48h at the same conditions. After that, viable bacteria were counted and determined the number of colonies forming units/ml (CFU/ml). The minimal lethal concentration (MLC) was obtained when the antimicrobial agents killed more than 50% of the tested microbial strains. All experiments were performed in triplicate.

Anti-biofilm activity

Dentin blocks preparation (Protocolo Anexo E)

This study was approved by the Animal Committee of Araçatuba Dental School, UNESP, Brazil (Protocol: 2014/00592 Anexo N). Bovine incisors were extracted and stored in a 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. Initially, roots were separated from crowns, using a diamond disc (KG Sorensen D 91, Barueri, SP, Brazil) at 1mm below the cementoenamel junction. 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, 102mm x0.3mmx12.7mm, Extec Corporation, Enfield, CT, USA) using a precision saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA) separated by a spacer disk (thickness 3mm) under refrigeration with distilled water. Root dentin slices were cut in four blocks with 3mm x 3mm x 0.7 mm. Dentin blocks (n = 10) were sequentially polished using 400-,600-,800- and 1,200-grade 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 with 17% EDTA for 3 min and deionized water for 5 min. After autoclaving for 15 minutes at 121 ° C, dentin blocks were stored at 4º C until use within one week (24).

Biofilm assays (Protocolo Anexo F)

Biofilms with *E. faecalis* and *F. nucleatum* strains were conducted testing the peptide that showed the best antimicrobial activity on MIC/MLC assays. Initially, dentin blocks (n = 10) were fixed with double sided tape at the bottom of 96-well microplates. 100µl of bacterial suspension in BHI with 0.5% glucose for E. faecalis or BHI supplemented with hemin and menadione plus 1% glucose for *F. nucleatum* were inoculated in each well in a final concentration of 1-5x10^5CFU/ml. After 48 h of incubation at 37°C in 5% CO_2 for *E. faecalis* and in anaerobic chamber for *F. nucleatum,* bacterial suspensions were aspirated and each dentin block was washed once with 100µl of 0.9% saline. After that, 100µl of the antimicrobial agents at the concentration of 5X and 10X MLC was placed in each well for 4 h and 24 h. After the period of incubation, each dentin block was washed twice with 100µl of 0.9% saline and aseptically removed from the bottom and inserted in microtube containing 500µl of 0.9% saline. For recovery of bacterial cells within the dentinal tubules, blocks were submitted to a

ultrasonic bath using deionized water for 20 min, followed by vortexing (AP 56, Phoenix) for 30 s and sonication during 30s at 30W (Misonix Ultrassonic Liquid Processors, model S-4000, USA). Specimens were vortexed again for 1 min and serially diluted. Each dilution was inoculated on BHIA plates for *E. faecalis* and Blood Agar for *F. nucleatum*. The plates were then incubated for 48 h and the number of CFU/ml was calculated. The experiments were performed in duplicate in two independent assays (25).

Confocal Laser Scanning Microscopy (CLSM) (Protocolo Anexo G)

Biofilm assays for CLSM analysis were conducted with E. faecalis testing the peptide that showed the best antimicrobial activity on MIC/MLC assays. Dentin blocks (n = 6) measuring 3mmx3mmx0.5 mm were inserted in wells, as methodology described above and biofilm was formed during 24h and exposed to peptide/CHX at 5X MLC concentration for 4h. After this period, dentin blocks were washed once with sterile deionized water and stained with 100 µl 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 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Two additional uninfected specimens were stained under the same protocol and used as negative control. Fluorescence from the stained cells was viewed by CLSM (Leica TCS SP5, Microsystems GmbH), using 63x 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-µm 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 cells ratios of the infected dentinal tubules, all scans were reconstructed in a three-dimensional model by the same software. The quantification of red fluorescence ratio in relation to green-and-red fluorescence was determined by software denominated Image J 1.48 (NIH, Bethesda, MA, USA) indicating the proportion of dead cells for each antimicrobial agent tested (25).

Statistical analysis

Data from cytotoxicity assays were expressed in means/standard deviation and submitted to ANOVA and Tukey tests in order to compare the effect of peptides and

CHX/APT on fibroblast cells, considering each concentration separately. Antimicrobial activity in planktonic conditions was determined considering the percentage of microbial reduction obtained by peptides and CHX in relation to control without these antimicrobial agents (100% growth), under minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC). Box-whisker plots were performed to represent the distribution of non-parametric data obtained in the *E. faecalis* biofilm assays, considering 4h and 24h of KR-12-a5 and CHX exposure. Mann-Whitney tests were applied to compare the anti-biofilm activity of KR-12-a5 and CHX, considering time of exposure (4 and 24h) and concentration of these antimicrobial agents (5x and 10x MLC). Quantification of dead cells in relation to total cells (dead cells/live plus dead cells) in dentin blocks exposed to KR-12-a5 and CHX were determined after analysis of images obtained from CLSM using software Image J (NIMH, Bethesda, Maryland, USA). CLSM data were converted in means/standard deviations and submitted to ANOVA and Tukey tests ($p \leq 0.05$) to compare the anti-biofilm action of the antimicrobial agents in dentin tubules. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analyses.

RESULTS

Cytotoxicity tests

LL-37 and hBD-3-1C^{V} affected cell metabolism in concentrations above 500µg/ml (less than 70% of cell growth). KR-12-a5 reduced its cytotoxicity in concentrations below 125 µg/ml, although there was no statistical difference between KR12-a5 and LL-37 for any concentration studied. KR-12-a5 and hBD-3-1C^{V} were statistically different 125-500µg/ml. APT influenced minimally cell growth at the concentrations below 125µg/ml. Among the antimicrobial agents tested, CHX had the effect more cytotoxic to L-929 cells in concentration higher than 15.62 µg/ml **(Figure 1)**.

Antimicrobial activity

Among the peptides, KR-12-a5 had the best bactericidal activity for both times of exposure, showing MIC values ranging 1.95µg/ml to 62.5µg/ml and MLC ranging 3.91 to 62.5µg/ml. LL-37 had antimicrobial action on *Streptococcus mutans, Actinomyces israelii, Fusobacterium nucleatum and Porphyromonas gingivalis* (MIC/MLC range: 3.91-1000µg/ml), but not for *Enterococcus faecalis* (MIC/MLC range: 1000-2000µg/ml).

Candida albicans growth was affected only for KR-12-a5 and CHX, considering the concentrations tested in the present study (below 4000µg/ml). Similar inhibitory effect was observed for hBD-3-1C^{V} and LL-37 against *S. mutans* and *A. israelii.* hBD-3-1C^{V} had lower effect against *F. nucleatum* (MIC/MLC: 1000µg/ml), however for *P. gingivalis,* hBD-3-1C^{V} had better MIC/MLC results (range: 31.25-62.5µg/ml). KR-12-a5 and CHX had markable antimicrobial effect in the non-toxic concentrations **(Table 1 and 2).**

Anti-biofilm activity

KR-12-a5 (5X and 10X MLC) reduced the percentage of bacterial reduction for both times of exposures. However, the effect of KR-12-a5 (5X and 10X MLC) on bacterial reduction was higher in 4h (30-40%) when compared to 24h (around 20%) of exposure. The same was observed for CHX (5X and 10X MLC) that showed superior bacterial reduction in 4h compared to 24h. CHX (10X) had statistically superior anti-biofilm effect compared to the other groups, including CHX 5x MLC, in 24h of exposure, similar to that observed for 4h. KR-12-a5 and CHX, in both concentrations (5x and 10x MLC) killed 100% of *F. nucleatum* biofilm after 4h and 24h of exposure **(Figure 2)**.

CLSM analysis

(Figure 3) KR-12-a5 (5x MLC) obtained similar anti-biofilm activity when compared to CHX (5x MLC) and both of them were superior to control, showing 60- 70% of dead cells inside the tubules. Figure 4 shows representative images obtained from *E. faecalis* biofilm on dentin blocks. KR-12-a5 **(Figure 4A)** showed slightly higher quantification of dead cells (red points) when compared to CHX **(Figure 4B)** and culture medium control **(Figure 4C)** which presented strong predominance of live cells (green points).

Discussion

Traditional chemical-mechanical treatments with or not subsequent application of medication with synthetic substances on root canal systems are currently indicated for most of the cases of endodontic infection. However, in young permanent teeth, biological materials with antimicrobial properties could be an alternative to control bacterial infection, preserving cell viability in apical region and completing root

formation. AMPs have been studied as biological alternatives to synthetic antibiotics in virtue of their broad range antimicrobial activity, including oral bacteria (26).

In this present study, analogues of recognized antimicrobial peptides were tested to evaluate if chemical changes in their sequence or structure could improve antimicrobial activity of original peptides without cause toxicity to host mammalian cells. This strategy aimed to reduce costs and difficulties with the peptide synthesis and the degradation by endogens factors. Due their cationic characteristic, AMPs have high affinity to anionic proteins such as albumin or glycosaminoglycans, naturally found in saliva, serum or other biological fluids, reducing their ability to bind to bacterial membranes. Besides, they may be degraded by bacterial or host proteases or become inactive by high concentrations of salts also present in these fluids (26).

Among the well-characterized human β-defensins, hBD-3 has been extensively studied because of its broad range antibacterial, antifungal and antiviral activities. These properties are not affected by high salts concentrations of monovalent and divalent cations found at biological fluids (7). However, the complex structure of hBD-3, characterized by six conserved disulfide-linked cystein residues make peptide synthesis highly expensive. Taylor et al. (14) synthesized the hBD-3 analogue, hBD-3-1C^{v} replacing five of the six cysteines by alanines, leaving on the fifth (C^{v}) cystein in the position and demonstrated that disulfide bonds were not important for antimicrobial activity and chemotaxis, but the presence of C^{v} was essential for attracting monocytes to inflammatory sites. LL-37 is another potent antimicrobial cationic peptide with several immunomodulatory functions, besides stimulate angiogenesis and re-epithelialization (8). This peptide could serve as the template for the development of new drugs using functional smaller fragments for microbiological and immunological applications. KR-12 (amino acids 18 to 29) is the smallest peptide region of LL-37 that still conserves antimicrobial activity similar to original peptide (15). In order to improve the antimicrobial action, Jacob et al. (16) synthesized analogues of KR-12, such as KR-12-a5, replacing some amino acids, like asparagin and glycin by lysines to increase positive charge and adding leucins to increase hydrophobic angle. KR-12-a5 had higher inhibitory activity against Gram positive and Gram negative bacteria, similar to results found in the present study and anti-endotoxic effect comparable to original LL-37.

One limitation of peptide use as future drugs is the toxicity of peptides on host cells at therapeutic concentration. LL-37 at high concentration (>13μM, approximately

60µg/ml) was toxic to human peripherical blood leukocytes and T-cell line (27) and hBD3 was toxic for human monocytic cell line above 50µg/ml. In the present study, peptides affected minimally fibroblast cell metabolism (L929) with more than 70% viable cells up to 250µg/ml and 125µg/ml for hBD-3-1C^{v}/LL-37 and KR-12-a5, respectively. Our results are according to Kluver et al. (28), which tested cytotoxicity of original hBD-3 with three dissulfide bonds and their analogues with replacement of cysteines by alanine or tryptophan. The authors demonstrated that the substitution of cysteines by alanines caused a reduction in the overall hydrophobicity become the hBD-3 analogues less cytotoxic in comparison to original hBD-3. In the study of KR-12 analogues, KR-12-a5 was toxic to macrophages cells at the concentrations above 12.5µM (approximately 60µg/ml). The authors explained that KR-12-a5 had the highest hydrophobicity among the KR-12 peptides causing higher cell toxicity, but increasing their LPS-neutralizing and antimicrobial effect. In human body, high concentrations of AMPs are controlled by their binding to biological fluids proteins, reducing cytotoxicity (29). All peptides tested were less cytotoxic than CHX solution in concentrations above 7.81µg/ml.

In the current study, peptide LL-37 and analogue hBD-3-1C^{v} have antimicrobial activity against the most of bacteria tested. hBD-3-1C^{v} had similar results on *S. mutans* and *A. israelii* and lower effect against *F. nucleatum* and *P. gingivalis* when compared to LL-37. Our findings corroborated with the results obtained by Ouhara et al. (12), except by *F. nucleatum*. They demonstrated excellent antimicrobial action of original hBD-3 and LL-37 against oral pathogens, including Gram-positive and Gram-negative bacteria, such as *A. actinomycetemcomitans, P. gingivalis, P. intermedia, F. nucleatum, S. mutans, S. sobrinus, S. salivarius, S. sanguinis, S. mitis* and *L. casei*.

Forms of hBD-3, biologically or synthetically produced, were also evaluated by some investigators. Song et al. (30) evaluated recombinant hBD-3 synthetic peptides (rhBD-3) looking for an alternative to reduce the cost of production of original hBD-3 and maintained or increasing antimicrobial effect of original peptide hBD-3. They observed that rhBD-3 was effective not only against *S. aureus* or *E. coli*, but also on some pathogenic microorganisms found in endodontic infections, such as *S. mutans, A. naeslundii, E. faecalis*, *C. albicans* and *F. nucleatum*. In our study, *E. faecalis* was more resistant to hBD-3-1C^{v}. Anti-*Candida albicans* activity was not found for hBD-3-1C^{v} different from the results found for the original peptide hBD-3 (13). The lower

antimicrobial effect of hBD-3-1-C^{v} compared with original hBD-3 could be related to reduction of its hydrophobicity by the replacement of cysteines by alanines (28). The first and only study that evaluated the antimicrobial activity of hBD-3-1C^{v} was developed by Taylor et al. (14) and they found superior antimicrobial activity against *P. aeruginosa* and *S. aureus.*

In this study, KR-12-a5 was the most effective peptide with strong antimicrobial activity against all bacteria tested (*S. mutans*, *A. israelli*, *E. faecalis*, *F. nucleatum*, and *P. gingivalis*). KR-12-a5 killed *C. albicans* at lower values of MIC (15.62-31.25µg/ml). Jacob et al. (16) evaluated antimicrobial activity of KR-12 and their analogues, including KR-12-a5, and they were similarly effective obtaining very lower MIC values against important pathogens.

Most studies testing antimicrobial activity of new substances have been usually conducted using minimal inhibitory tests, determining bacteriostatic or bactericidal effect under planktonic conditions. However, microorganisms form communities in a complex environment, called biofilms, which requires highly concentrated and more powerful antimicrobials. In this study, peptide with the best antimicrobial effect, KR-12-a5, was tested against biofilm of *E. faecalis* and *F. nucleatum.* The original peptide LL-37 and its fragments have shown their action in breaking three-dimensional structures of *P. aeruginosa* biofilms (31) and *Acinetobacter baumannii* (32). No study was found evaluating the anti-biofilm effect of KR-12-a5. Both CHX and KR-12-a5 were effective to eradicate *F. nucleatum*, at the concentrations tested in this study. However, the same was not observed for *E. faecalis.* Both antimicrobials agents reduced 30-40% of *E. faecalis* biofilm. KR-12-a5 was similar to CHX in reducing *E. faecalis* biofilm on dentin blocks at 5x MLC for both times of exposure. This result was also observed for CLSM analysis showing high percentage of dead cells for both antimicrobial agents (KR-12-a5 and CHX). CHX had superior effect in comparison to KR-12-a5 at the concentration of 10X MLC, with 24h of exposure. Nagant et al. (31) demonstrated reduction of more than 50% of *P. aeruginosa* biofilm by CLSM only at high concentration of LL-37(50 µM), original form of KR-12.

Overall, 4h of peptides exposure, both planktonic and biofilm conditions had better results in comparison to 24h. This result suggested that peptides could be naturally degraded with the time. Therefore, in order to maintain chemical and physical

stability and provide slow release, drug-delivery systems could be an attractive strategy for the administration of peptides (33).

Conclusion

KR-12-a5 had superior antimicrobial activity and showed anti-biofilm activity similar to CHX at non-cytotoxic concentrations. Therefore, KR1-12-a5 could be a potential antimicrobial agent for endodontic application.

Acknowledgements

This study was supported by FAPESP (grant #2013/24606-5), PROPe/CDC-UNESP (grant # 0403/001/14) and CNPq (grant # 134551/2013-3).

References

1. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004 Nov 1;15(6):348-81.
2. Byström A, Sundqvist G (1981). Bacteriological evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 89:321-328.
3. Chávez De Paz LE, Dahlén G, Molander A, Möller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J. 2003 Jul;36(7):500-8.
4. Iglesias-Linares A, Yáñez-Vico RM, Sánchez-Borrego E, Moreno-Fernández AM, Solano-Reina E, Mendoza-Mendoza A. Stem cells in current paediatric dentistry practice. Arch Oral Biol. 2013 Mar;58(3):227-38.
5. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009 Nov;88(11):969-81. doi: 10.1177/0022034509346549. Appendix
6. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Micros 2003; Dec 36(4);247-252.
7. Wiesner J, Vilcinskas A. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 2010 Sep/Oct;1(5):440-464.

8. Vandamme D, Landuyt B, Luyten W, Schoofs L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell Immunol. 2012 Nov;280(1):22-35. doi: 10.1016/j.cellimm.2012.11.009. Epub 2012 Nov 29. Review.
9. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. Int Arch Allergy Immunol. 2006; 140(2):103-12. Epub 2006 Mar 24.
10. Chotjumlong P, Bolscher JG, Nazmi K, Reutrakul V, Supanchart C, Buranaphatthana W, Krisanaprakornkit S. Involvement of the P2X7 purinergic receptor and c-Jun N-terminal and extracellular signal-regulated kinases in cyclooxygenase-2 and prostaglandin E2 induction by LL-37. J Innate Immun. 2013;5(1):72-83. doi: 10.1159/000342928. Epub 2012 Oct 23.
11. Jönsson D, Nilsson BO. The antimicrobial peptide LL-37 is anti-inflammatory and proapoptotic in human periodontal ligament cells. J Periodontal Res. 2012 Jun;47(3):330-5.
12. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M. Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, {beta}-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother. 2005 Jun;55(6):888-96.
13. Wong JH, Ng TB, Legowska A, Rolka K, Hui M, Cho CH. Antifungal action of human cathelicidin fragment (LL13-37) on *Candida albicans*. Peptides. 2011 Oct;32(10):1996-2002. doi: 10.1016/j.peptides.2011.08.018. Epub 2011 Aug 26.
14. Taylor K, Clarke DJ, McCullough B, Chin W, Seo E, Yang D, Oppenheim J, Uhrin D, Govan JR, Campopiano DJ, MacMillan D, Barran P, Dorin JR. Analysis and separation of residues important for chemoattractant and antimicrobial activities of beta-defensin3. J Biol Chem. 2008 Mar 14;283(11):6631-9.
15. Wang G.Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J Biol Chem. 2008 Nov 21;283(47):32637-43.
16. Jacob B, Park IS, Bang JK, Shin SY.Short KR-12 analogs designed from human cathelicidin LL37 possessing both antimicrobial and antiendotoxic activities without mammalian cell toxicity. J Pept Sci. 2013 Nov;19(11):700-7.

17. Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y. Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. J Periodontal Res. 2007 Oct;42(5):410-9.
18. Merrifield, R.B., 1963. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide, J. Am. Chem. Soc. 2149–2154.
19. Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34, 595-598.
20. Bedran TB, Mayer MP, Spolidorio DP, Grenier D. Synergistic anti-inflammatory activity of the antimicrobial peptides human beta-defensin-3 (hBD-3) and cathelicidin (LL-37) in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. PLoS One. 2014 Sep 4;9(9):e106766. doi: 10.1371/journal.pone.0106766. e Collection 2014.
21. NCCLS: National Committee for Clinical Laboratory Standards, 2002a. Reference method for broth dilution antifungal susceptibility testing of yeast: approved standard. National Committee for Clinical Laboratory Standards, Wayne, PA, CLSI document M27-A2.
22. NCCLS: National Committee for Clinical Laboratory Standards, 2002b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th ed., Wayne, PA, National Committee for Clinical Laboratory Standards, CLSI document M7-A5.
23. Mor A, Hani K, Nicolas PJ. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 1994; 269:31635–41.
24. Liu H, Wei X, Ling J, Wang W, Huang X. Biofilm formation capability of *Enterococcus faecalis* cells in starvation phase and its susceptibility to sodium hypochlorite. J Endod. 2010 Apr;36(4):630-5. doi: 10.1016/j.joen.2009.11.016. Epub 2010 Feb 6.
25. Li W, Liu H, Xu Q. Extracellular dextran and DNA affect the formation of *Enterococcus faecalis* biofilms and their susceptibility to 2%chlorhexidine. J Endod. 2012 Jul;38(7):894-8.
26. Batoni G, Maisetta G, Brancatisano FL, Esin S, Campa M. Use of antimicrobial peptides against microbial biofilms: advantages and limits. Curr Med Chem. 2011;18(2):256-79. Review.

27. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J Biol Chem. 1998 Feb 6;273(6):3718-24.
28. Klüver E, Schulz-Maronde S, Scheid S, Meyer B, Forssmann WG, Adermann K. Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. Biochemistry. 2005 Jul 19;44(28):9804-16.
29. Ciornei CD, Sigurdardóttir T, Schmidtchen A, Bodelsson M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrob Agents Chemother. 2005 Jul;49(7):2845-50.
30. Song W, Shi Y, Xiao M, Lu H, Qu T, Li P, Wu G, Tian Y.In vitro bactericidal activity of recombinant human beta-defensin-3 against pathogenic bacterial strains in humantooth root canal. Int J Antimicrob Agents. 2009 Mar;33(3):237-43.
31. Nagant C, Pitts B, Nazmi K, Vandenbranden M, Bolscher JG, Stewart PS, Dehaye JP. Identification of peptides derived from the human antimicrobial peptide LL-37 active against biofilms formed by *Pseudomonas aeruginosa* using a library of truncated fragments. Antimicrob Agents Chemother. 2012 Nov;56(11):5698-708. doi: 10.1128/AAC.00918-12. Epub 2012 Aug 20.
32. Feng X, Sambanthamoorthy K, Palys T, Paranavitana C.The human antimicrobial peptide LL-37 and its fragments possess both antimicrobial and antibiofilm activities against multidrug-resistant *Acinetobacter baumannii*. Peptides. 2013 Nov;49:131-7. doi: 10.1016/j.peptides.2013.09.007. Epub 2013 Sep 23.
33. Dailey LA, Wittmar M, Kissel T. The role of branched polyesters and their modifications in the development of modern drug delivery vehicles. J Control Release. 2005; 101(1-3):137-49.

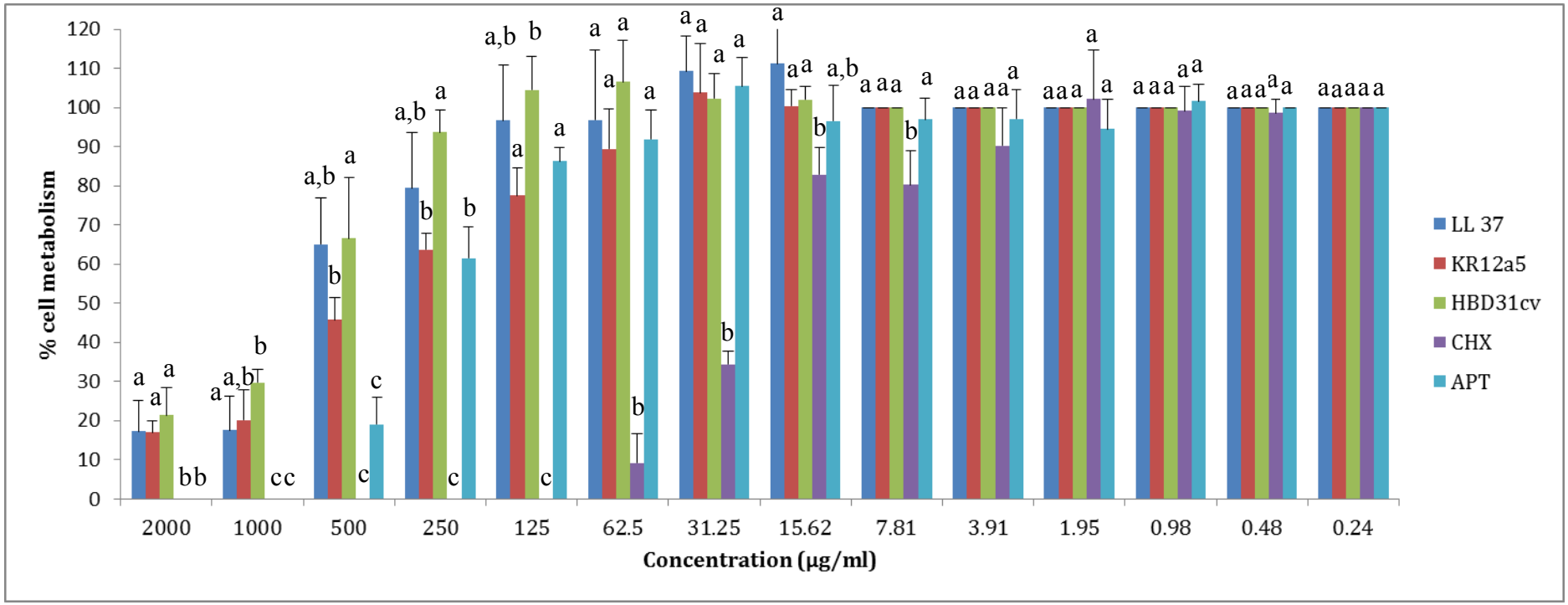


Figure 1. Comparison of toxicity in L-929 cells post treatment with different concentrations of peptides, chlorhexidine (CHX) and amphotericin (APT). Means (bars = standard deviation) of percentage of cell metabolism (MTT assay) after antimicrobial agents exposure.

[a] Different lower letters show statistical difference among the groups, considering each concentration separately, according to ANOVA and Tukey tests.

Table 1. Antimicrobial activity of peptides and CHX after 4h of exposure, in planktonic conditions

		LL-37	KR-12-a5	HBD-3-1C^{v}	CHX
S. mutans	**MIC***	250	3.91	250	0.03
	% microbial reduction**	16.64± 9.74	100	33.05± 19.05	15.28± 2.3
	MLC†	1000	3.91	250	7.81
	% microbial reduction‡	100	100	100	100
A. israelii	**MIC**	3.91	1.95	7.81	0.48
	% microbial reduction	100	37.56± 4.14	100	21.87± 6.55
	MLC	3.91	3.91	7.81	0.98
	% microbial reduction	100	100	100	100
E. faecalis	**MIC**	1000	31.25	500	3.91
	% microbial reduction	100	66.11± 8.74	54.73± 11.52	51.12± 3.64
	MLC	1000	31.25	> 4000	15.62
	% microbial reduction	100	66.11± 8.74	-	100
C. albicans	**MIC**	> 4000	15.62	> 4000	7.81
	% microbial reduction	-	100	-	100
	MLC	> 4000	15.62	> 4000	7.81
	% microbial reduction	-	100	-	100

* MIC – minimal inhibitory concentration (µg/ml)
** Percentage of microbial reduction in relation to control without antimicrobial agents (100% growth), under MIC concentration (µg/ml)
† MLC – minimal lethal concentration (µg/ml)
‡ Percentage of microbial reduction in relation to control without antimicrobial agents (100% growth), under MLC concentration (µg/ml)

Table 2. Antimicrobial activity of peptides and CHX after 24 to 72h of exposure, in planktonic conditions

		LL-37	KR-12-a5	HBD-3-1C[v]	CHX
S. mutans	**MIC***	250	3.91	250	0.24
	% microbial reduction**	47.97± 8.45	50.45± 45.52	100	46.35± 4.84
	MLC†	1000	7.81	250	7.81
	% microbial reduction‡	100	100	100	100
A. israelii	**MIC**	7.81	3.91	7.81	0.98
	% microbial reduction	100	100	100	100
	MLC	7.81	3.91	7.81	0.98
	% microbial reduction	100	100	100	100
E. faecalis	**MIC**	2000	62.5	500	7.81
	% microbial reduction	100	100	20.45± 1.04	49.42± 23.55
	MLC	2000	62.5	> 4000	15.62
	% microbial reduction	100	100	-	100
C. albicans	**MIC**	> 4000	15.62	> 4000	7.81
	% microbial reduction	-	31.12± 1.04	-	100
	MLC	> 4000	31.25	> 4000	7.81
	% microbial reduction	-	100	-	100
F. nucleatum[#]	**MIC**	250	7.81	1000	1.95
	% microbial reduction	100	100	100	100
	MLC	250	7.81	1000	1.95
	% microbial reduction	100	100	100	100
P. gingivalis[##]	**MIC**	7.81	3.91	31.25	7.81
	% microbial reduction	100	100	57.37± 8.5	43.7± 13.22
	MLC	7.81	3.91	62.5	7.81
	% microbial reduction	100	100	100	77.07± 19.9

* MIC – minimal inhibitory concentration (µg/ml)
** Percentage of microbial reduction in relation to control without antimicrobial agents (100% growth), under MIC concentration (µg/ml)
† MLC – minimal lethal concentration (µg/ml)
‡ Percentage of microbial reduction in relation to control without antimicrobial agents (100% growth), under MLC concentration (µg/ml)
[#] 48 h of bacterial exposure
[##]72h of bacterial exposure

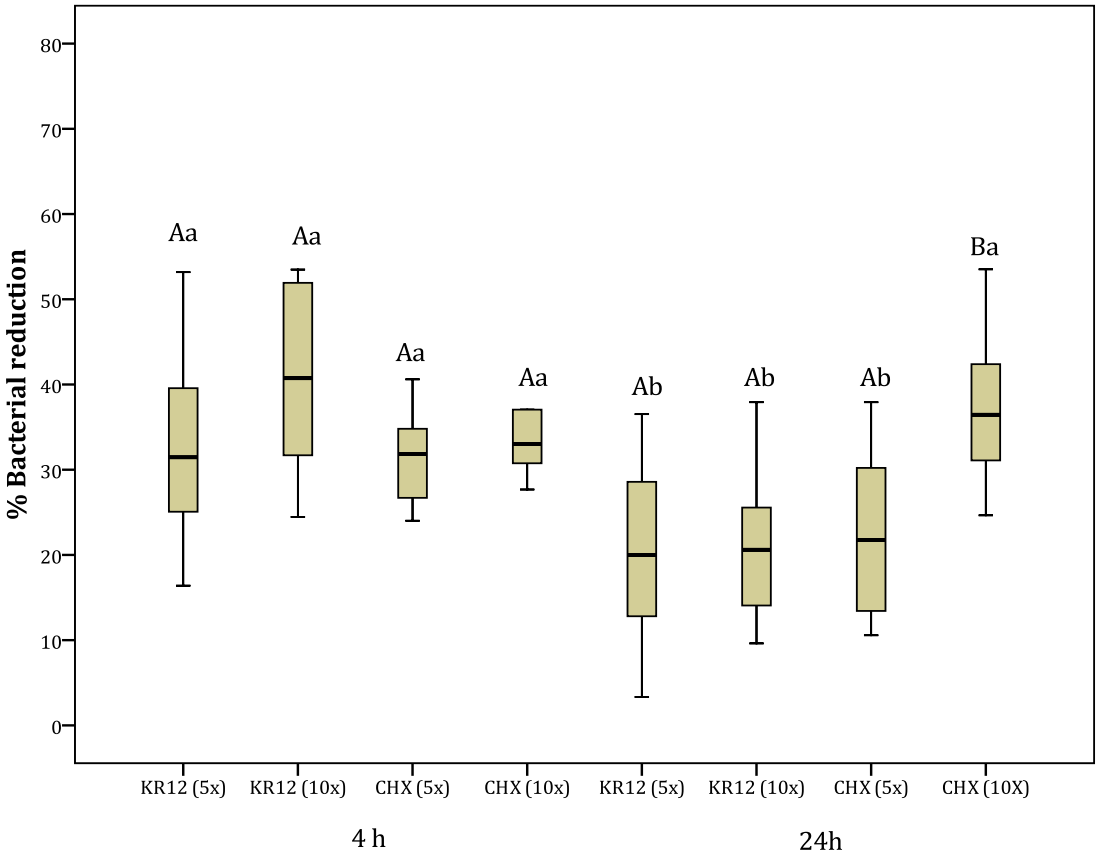


Figure 2. Box-whisker plots of the anti-biofilm activity of KR-12-a5 and CHX against *E. faecalis*.
Bars indicate minimum and maximum values. Boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median.

[A] Different upper case letter show statistical differences among the groups, considering each time separately, according to Mann-Whitney test ($p \leq 0.05$)
[a] Different lower case letter show statistical differences among the groups, comparing 4h and 24h, according to Mann-Whitney test ($p \leq 0.05$)
KR12 – KR-12-a5
CHX – chlorhexidine

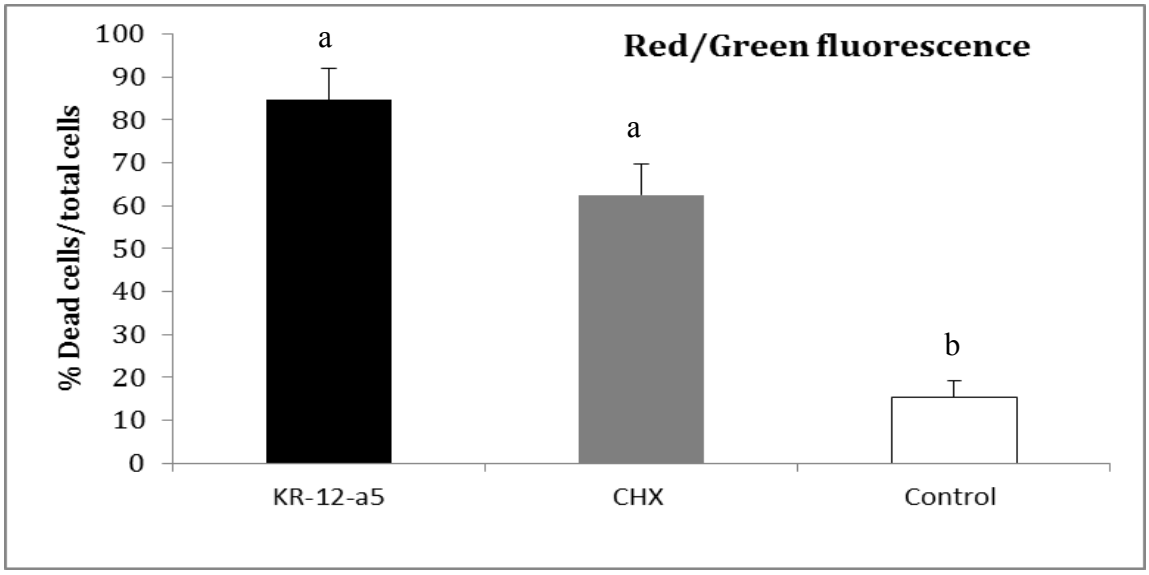
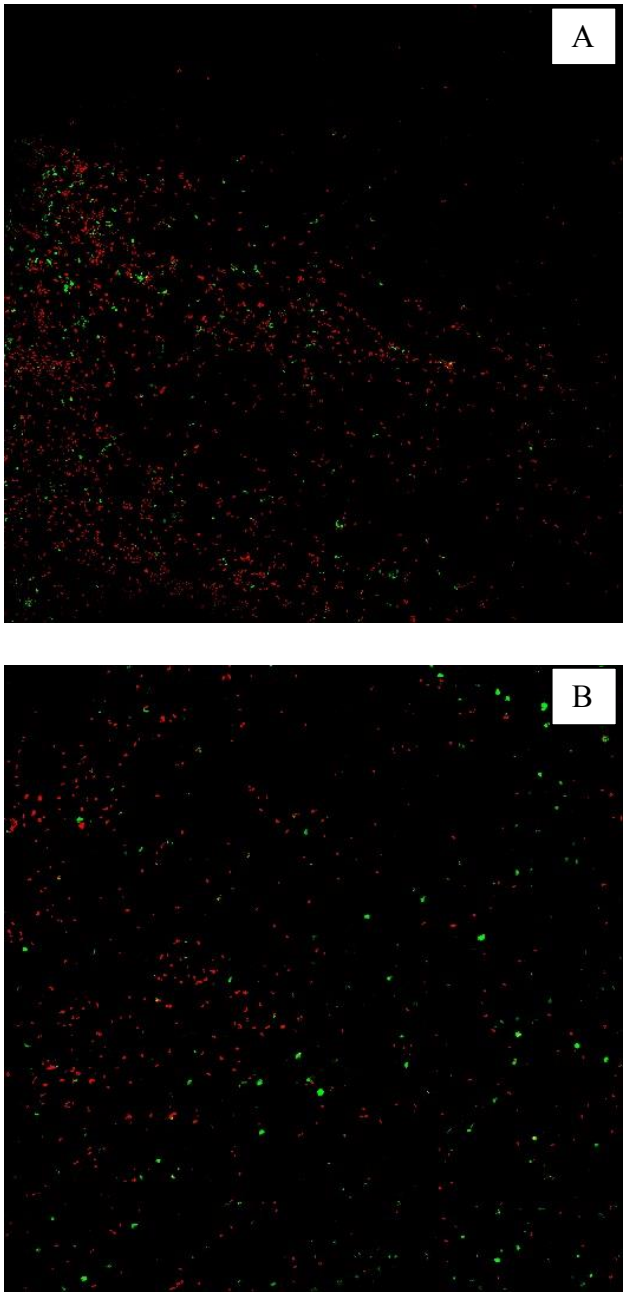


Figure 3. Means (bars-standard deviations) of percentage of dead cells obtained after CLSM analysis of *E. faecalis* biofilm.

[a] Different lower case letter show statistical differences among the groups, according to ANOVA/Tukey tests ($p \leq 0.05$)
CHX – chlorhexidine
Control – bacterial culture without antimicrobial agents

Figure 4. Representative CLSM images (63x) from *E. faecalis* biofilm inside dentin blocks, under effect of KR-12-a5 (A), CHX (B) and culture medium (C).



Capítulo 2

Karina Sampaio Caiaffa

Effect of analogues of cationic peptides on phenotypic and genotypic markers of mineralization in MDPC-23 odontoblast-like cells

***The manuscript is according to the guide for authors of Journal of Endodontics (Anexo A).**

Karina Sampaio Caiaffa[1], Fernanda Gonçalves Basso[2], Norival Alves Santos-Filho[3], Carlos Alberto de Souza Costa[2], Eduardo Maffud Cilli[3], Cristiane Duque[4]*****

[1] Department of Endodontics, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba,São Paulo, Brazil.

[2] Department of Physiology and Pathology, Araraquara Dental School, Univ Estadual Paulista (UNESP),Araraquara,São Paulo, Brazil.

[3]Department of Biochemistry and Chemical Technology, Institute of Chemistry, UNESP, Univ Estadual Paulista, Araraquara, São Paulo, Brazil

[4] Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, São Paulo, Brazil.

***Corresponding author:**

Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)

Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil

Tel: (+55) 1836363315

E-mail: cristianeduque@yahoo.com.br, cduque@foa.unesp.br

The authors declare that have no conflicts of interest.

Abstract

Introduction: The aims of the study were to evaluate cell viability and the effect of analogues of cationic peptides on phenotypic and genotypic markers of mineralization in odontoblast-like cells.

Methods: MDPC-23 odontoblast-like cells were exposed to serial dilutions of peptides LL-37, hBD-3-1C^{V} and KR-12-a5 and cell viability was evaluated by MTT assays. Phenotypic analysis of total protein (TP) production, alkaline phosphatase (ALP) activity and mineralized nodule deposition assays were carried out after exposure to different concentrations of peptides (0.78 - 62.5 μg/ml) for 24 h followed by changes of osteogenic medium for 7 days. The gene expression of mineralization markers (DSPP and DMP-1) was performed by quantitative PCR, after 24h of peptide exposure and incubation for 14 days in osteogenic medium change.

Results: LL-37 and hBD-3-1C^{V} affected minimally cell growth at the concentrations below 62.5 μg/ml. KR-12-a5 was less cytotoxic below 31.25 μg/ml. TP production was similar for all groups compared with the control group, except by hBD-3-1C^{V} (15.62 μg/ml). LL-37 (62.5 μg/ml) induced higher ALP activity than control and another groups. LL-37 and hBD-3-1C^{V}, at 62.5 μg/ml and KR-12-a5 at 31.25 μg/ml stimulated the highest deposition of mineralized nodules. Considering DSPP and DMP-1 expression, no statistical differences were observed among the groups or when they were compared with the control.

Conclusion: KR-12-a5 stimulated initial mineralized nodules deposition, similar to another peptides, but in lower and minimally cytotoxic concentration. None peptides influenced the expression of DSPP and DMP-1, genes involved in active dentin mineralization.

Keywords: Cationic Antimicrobial Peptides, Cytotoxicity, Cell Culture, Polymerase Chain Reaction, Endodontics.

Introduction

Endodontic diseases occur as a result of the autogenous oral microbiota invasion inside system of root canals and perirradicular tissues. Consequently, the first step of the endodontic therapy must be the elimination of microorganisms and prevention of root canal reinfection (1). Due the complex system of lateral and accessories channels and the persistence of some microbial species, the chemical-mechanical treatment is not enough to promote complete disinfection of root canal system, become the use of an intracanal medication with antimicrobial properties indispensable (2, 3). Natural or synthetic antimicrobials have been studied as alternative agents to traditional intracanal medications, in order to eliminate bacteria and their toxins and not cause damage to the adjacent periapical tissues, leading to tissue regeneration and complete apical root formation in immature permanent teeth (4, 5).

Antimicrobial cationic peptides (AMPs) have been attracted attention in Medicine and Dentistry because of their wide-range inhibitory activity against several bacterial, viral and fungal species (6, 7). AMPs represent a family of peptides found in various sites of the human body, including oral cavity. In order to maintain normal microbiota in steady state, besides their antimicrobial action, they also promote modulation of host immune responses (8, 9). The main AMPs present in saliva and crevicular gingival fluid are defensins and cathelicidins. The α and β-defensins have immunomodulatory function by modifying the cell migration and maturation inducing cytokines and the release of histamine and prostaglandin A2 of mastocytes (8, 9, 10). The human cationic peptide (hCAP-18) is the only cathelicidin identified in humans isolated firstly in neutrophil granules. After secretion, hCAP-18 is broken by the action of proteases in one long chain active peptide called LL-37 and two smaller peptide fragments. LL-37 is a multifunctional peptide with antimicrobial function and ability to modulate innate immunity, besides to stimulate angiogenesis, skin healing and chemotaxis (11).

In virtue of high cost of AMPs production, several studies have proposed chemical modifications or reduction in the peptides length, conserving or improving their antimicrobial and immunoregulatory activity. Taylor et al. (12) chemically modified the original hBD-3, replacing 5 of the 6 cysteines by alanines and eliminating disulfide bonds among them and found that the hBD-3 analogue called hBD-3-1C^{v} conserved its antimicrobial activity against *P. aeruginosa* and *S. aureus* and chemotactic effect on monocytes and T cells. Studies have investigated several properties of the smallest

fragment of LL-37 with antimicrobial activity, KR-12 (13, 14). Eight analogues of KR-12 (KR-12-a1 to KR-12-a8) were designed and microbiologically tested by Jacob et al. (14). The most of KR-12 analogues exhibited potent antimicrobial activity against strains of methicillin-resistant *S. aureus* and LPS-neutralizing action, without causing toxicity to eukaryotic cells. One of these analogues, KR-12-a5, had the highest anti-endotoxic activity, similar to original LL-37.

In addition to antimicrobial and anti-inflammatory action, an intracanal medication indicated to immature permanent teeth should induce residual epithelial Hertwig sheath and promote the differentiation of stem cells into odontoblasts or induce remaining odontoblasts deposit dentin and the natural closure of the root apex. In vitro studies have showed that LL-37 activates EGFR (epithelial growth factor receptor) and JNK (C-terminal kinase c-Jun), molecules involved in cell migration and proliferation in human dental pulp cells, demonstrating that these peptides could contribute to pulp regeneration (15). The β-defensins have also been shown to stimulate the differentiation of odontoblasts and stimulate dentin sialophosphoprotein (DSPP) and osteopontin (OPN), markers of mineralization expressed by dental pulp-derived cells (16). Additionally, proliferation, differentiation and biomineralization by osteoblast-like cells have also been documented for β-defensins by increasing of alkaline phosphatase (ALP) activity and expression of osteogenic markers such as BMP-2 and BMP- 4 (17). Monocytes treated with LL-37 differentiated into cells able to form bone and express intracellular proteins such as osteocalcin (OC), osteonectin (ON), bone sialoprotein II (BSP II), osteopontin (OP) and others (18). Kittaka et al. (19) showed that LL-37 was able to induce angiogenesis and regulate the recruitment of stem cells to promote bone regeneration in calvarian defect model in rats.

The aims of the study were to evaluate cell viability and the effect of analogues of cationic peptides on phenotypic (alkaline phosphatase activity, total protein production and mineralized nodules formation) and genotypic (DSPP and DMP-1 gene expression) markers of mineralization in odontoblast-like cells.

Materials and methods

Peptides synthesis (Análise da síntese dos peptídeos em Anexo B)

The peptides used in this study and their amino acid sequence were: cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRI-TES) (20), KR-12-a5 (LL-37 analogue - KRIVKLILKWLR-NH2) (14) and hBD-3-1C^{V} (human β-defensin-3 analogue - GIINTLQKYYARVRGGRAAVLSALPKEEQIGKASTRGRKCARRKK) (12). All peptides were synthesized at Institute of Chemistry, UNESP, Araraquara, SP, Brazil. Solid phase peptide synthesis was performed manually by the method previously described by Merrifield (21) using Fmoc (9-fluorenylmethyloxycarbonyl) protocols on the Rink-amide resin. The Fmoc group was deprotected by 20% piperidine/dimethylformamide (DMF). Coupling was performed at a two-fold excess over the amino component in the resin using diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt) in a solution of 50% (v/v) DCM (dichloromethane) and DMF. After 2 h of incubation, the coupling was assessed by the ninhydrin test (22). Cleavage from the resin and removal of the protecting groups from the amino acid residue side chains were performed simultaneously with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) for LL-37 and KR-12-a5 and 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% 1,2-ethanedithiol (EDT) and 1% triisopropylsilane (TIS) for hBD-3-1C^{v}, both for 2 h. After this procedure, the crude peptides were precipitated with anhydrous ethyl ether and separated from the non-soluble material by centrifugation. Subsequently, the peptides were extracted with 0.045% (v/v) TFA/H_2O (solvent A) and lyophilized for further purification. Molecular masses of peptides were estimated by mass spectrometry, using positive ion-mode electrospray ionization (ESI) apparatus (Bruker, Germany) and were in agreement with corresponding calculated values. Purification of synthesized peptides was performed in a semi-preparative HPLC Beckman System Gold on a reverse phase C18 column (2.1 × 25 cm, Phenomenex, Torrance, CA, USA). Final purity levels of peptides were determined at least 95% on a Shimadzu chromatography equipped with an analytical C18 reverse phase column (0.46 × 25 cm, Kromasil, Bohus, Sweden).

Preparation of antimicrobial agents

Peptides were weighed in an analytical balance (OHAUS Adventurer, Parsippany, NY, USA) at the concentration of 4 mg/ml and dissolved in sterile deionized water, except by hBD-3-1C^{V} which was dissolved in 0.045% (v/v) TFA/H_2O. All solutions were filtered with 0.2µm syringe filters (Kasvi, Curitiba, PR, Brazil).

MDPC-23 cell culture (Protocolo Anexo I)

The odontoblast-like cells (MDPC-23 - *mouse* dental papilla cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and containing 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO2 and 95% air at 37^{o}C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). Cells were sub-cultured at every 2 days until an adequate number of cells were obtained for the study.

Firstly, cells were seeded (1 X 10^{4} cells/well) onto sterile 96-well plates, which were maintained in the same conditions at 37^{o}C for 24 h. Experimental groups were then determined: control group (DMEM – no treatment), LL-37, hBD3-1C^{v} and KR-12-a5 at concentrations ranging from 125 to 0.78 µg/ml. 0.045% TFA acid solution used as solvent to stabilize HBD3-1C^{v} peptide was also tested in contact to cells. After cell growth, culture medium was aspirated and replaced by fresh FBS-free DMEM with the antimicrobial agents and incubated at 37^{o}C for 24h (23). One first assay was conducted with peptides at the concentrations described above to establish the best concentrations of peptides for subsequent analysis. After that, all assays were performed with peptides ranging from 62.5 to 0.78 µg/ml for 24h and subsequent osteogenic culture medium changes for 7 days (23, 24).

Analysis of cell viability (MTT assay)

Methyltetrazolium (MTT) assay is method determines the activity of succinate dehydrogenase - SDH enzyme - representing cell (mitochondrial) respiration and considered as the metabolic rate of cells. After incubation of cells in contact with DMEM (control group) or DMEM containing peptides (experimental groups), the culture medium was aspirated and replaced by 90 µL of fresh FBS-free DMEM and 100 µL of MTT solution (5 mg/ml in PBS) and cells were incubated at 37^{o}C for 4 h. After

incubation, culture medium with the MTT solution was aspirated and 100 µL of acidified isopropanol solution (0.04 N HCl) was added in each well to dissolve the violet formazan crystals resultant from the cleavage of the MTT salt ring by the SDH enzyme activity, producing a homogenous violet solution. Cell viability was evaluated using a spectrophotometer (Synergy H1 Hybrid Multi-Mode Microplate Reader – BioTek Instruments Inc, Winooski, USA) at 570 nm wavelength. The mean of absorbance values obtained from the control group (DMEM) was calculated and considered as 100% of cell viability and the percentage values for tested groups (n = 6) were calculated based on this criterion (23, 24).

Total protein (TP) production (Protocolo Anexo J)

Total protein production was performed in parallel with the alkalin phosphatase assay in 96- well culture plate (TPP – Techno Plastic Products AG 92096, Switzerland). After 24 hours of incubation with the peptides and control groups (n = 6), the culture medium was aspirated and cells were kept in osteogenic medium for additional 7 days (DMEM whit 10% SFB, supplemented with 50 µg/mL $^{-1}$ sodium ascorbate and 10 nmol L $^{-1}$ β-glycerophosphate; Sigma/Aldrich Corp.)

An amount of 150 µL of 0.1% sodium lauryl sulphate detergent in deionized water (Sigma / Aldrich Corp., St. Louis, MO, USA) were added to each well and maintained for 40 min at room temperature to produce cell lysis. Then, 100uL of this solution were used for total protein production analysis. After lysis, 100 µL of Lowry reagent solution (Sigma / Aldrich Corp., St. Louis MO, USA) were added to all samples. After 20 min at room temperature, 50 µL of Folin-Ciocalteau's phenol reagent solution (Sigma / Aldrich Corp., St. Louis MO, USA) were added to each well. Thirty minutes later, the absorbance of samples was measured at 655 nm wavelength by spectrophotometry. Total protein production was calculated from a standard curve using bovine serum albumin (BSA) concentrations (24).

Alkaline phosphatase (ALP) activity assay (Protocolo Anexo K)

The test was performed according to the instructions of the Kit's manufacturer. ALP activity was analyzed using the colorimetric endpoint assay (ALP Kit, reference 40; Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil) (n = 6). This test uses thymolphthalein

monophosphate substrate, a phosphoric acid ester substrate which is hydrolyzed by ALP releasing thymolphthalein. The kit measures the product of hydrolysis, altering the pH. The pH interrupted the ALP enzymatic activity and provides blue color to the solution that is directly proportional to the resulting enzymatic activity and is analyzed by spectrophotometer.

After 24 hours of incubation with the peptides and control(osteogenic medium) groups, the culture medium was aspirated and cells were cultured in osteogenic medium for additional 7 days (DMEM whit 10% SFB, supplemented with 50 μg/mL $^{-1}$ sodium ascorbate and 10 nmol L $^{-1}$ β-glycerophosphate; Sigma/Aldrich Corp.). An amount of 50 μL of cell lysate obtained from the same samples used for total protein production were used for ALP activity analysis. The absorbance was measured at 590 nm with a spectrophotometer (Synergy H1). ALP activity was calculated by a standard curve using known concentrations of the enzyme (24).

Mineralized nodule deposition (alizarin red staining) (Protocolo Anexo L)

After 24 h of incubation with the peptides and control groups, cells were cultured for 7 days in osteogenic medium (DMEM with 10% SFB, supplemented with 50 μg/mL $^{-1}$ sodium ascorbate and 10 nmol L $^{-1}$ β-glycerophosphate; Sigma/Aldrich Corp.) (n = 6). Cells were fixed with 70% ethanol for 1 h at 4 ° C, washed one time with deionized water and then stained with 100 μL / well of Alizarin Red dye (40 mmol L $^{-1}$; pH 4,2; Sigma-Aldrich Corp.) for 20 min, under gentle shaking (VDR Shaker, Biomixer, Ribeirão Preto, SP, Brazil). After aspiration of unincorporated dye, the cells were washed one time with deionized water and incubated with 200 μL / well of 10% cetylpyridinium chloride (Sigma/Aldrich Corp.) for 15 min under agitation to solubilize the nodules. The absorbance of the resulting solution (n = 6) was measured at 562 nm using a spectrophotometer (Synergy H1). The resulting absorbance was converted into percentages and the mean of the control group was calculated and considered as 100% staining of calcium deposits after 7 days, and the percentage values for the tested groups were calculated based on this criterion (24).

Gene expression of odontoblastic markers of dentin mineralization (Protocolo Anexo M)

RNA isolation and cDNA synthesis

For PCR analysis, MDPC-23 cells were exposed to antimicrobial agents for 24h and then incubated with osteogenic medium for 14 days (n = 6). Culture medium was replaced at every 24 hours. After 14 days, DMEM was aspirated and 100 µL of Lysis Solution (Ambion, Life Technologies, USA) were added to each sample and stored in microtubes at - 20 ° C. RNA isolation was obtained using specific Kits (RNAqueous®-Micro Total RNA Isolation Kit # AM1931, Life Technologies, USA). Briefly, 100 µL of lysate was added to 50 µL of 100% ethanol (Sigma Aldrich) and vortexed. The lysate / ethanol mixture were placed in the Micro Filter Cartridge Assembly and centrifuged (12.000rpm, 10 seconds, at room temperature). Then, 180 µL of Wash Solution 1 (Ambion, Life Technologies, USA) was added and samples were submitted to new centrifugation. Samples were washed twice with 180 µL of Wash Solution 2/3 Concentration (Ambion), followed by centrifugation and filters were transferred to Micro Elution Tubes with 10µL of Elution solution (Ambion) preheated at 75°C, and all samples were centrifuged (12.000rpm, 5 minutes). RNA concentration was determined using a spectrophotometer (Take 3 System, Synergy H1). cDNA was synthesized from each RNA sample for subsequent qPCR analysis using specific kits (High Capacity cDNA Reverse Transcriptions Kit, Applied Biosystems, Foster City, CA, USA), according to the following protocol. In a microcentrifuge tube, 10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix, MultiScribe Reverse Transcriptase and 0.5 µg/µl of the RNA of each sample were added. The samples were then submitted to the following amplification cycling conditions: 25^{o}C (10 min), 37^{o}C (120 min) and 85^{o}C (5 min) and frozen at -20^{o}C until use.

qPCR analysis

After cDNA synthesis, expression of the following odontoblastic markers of dentin mineralization: mouse dentine matrix phosphoprotein 1 (DMP-1) and mouse dentine sialophosphoprotein (DSPP) was assessed by quantitative-PCR (qPCR - Real Time PCR). Specific primers were synthesized from the mRNA sequence: DMP-1 FORWARD: 5'-CTGTGCTCTCCCAGTTGCCA-3 and REVERSE: 5'-GGTCACTATTTGCCTGTCCCTCT-3', and DSPP: FORWARD: 5'-

CGGAGGCTTTGAAGACATTGATTAC-3' and REVERSE: 5'-GCAGTTCCTGGATGTGTTAGAAGAG-3'. The qPCR reactions were prepared with standard reagents (Power SYBR Green PCR Master Mix, Applied Biosystems, Life Technologies, UK) and performed using Step One Plus Termocycler (Applied Biosystems) and CT values were analyzed by Step One Plus Software. The CT values for each sample were normalized by the endogenous control gene: glyceraldehyde-3-phosphatedehydrogenase (GAPDH) with primers sequences: FORWARD: 5'-AATGGTGAAGGTCGGTGTGAAC-3' and REVERSE: 5'-CGTGAGTGGAGTCATACTGGAAC-3' (25).

Statistical analysis

Data obtained from MTT assays were expressed in means (standard deviations) of percentage of cell viability in relation to control (DMEM - 100% of cell growth) and submitted to ANOVA/Tukey tests ($p \leq 0.05$), aimed to compare groups of peptides, considering each concentration separately or compare the same peptide at different concentrations. Data from alkaline phosphatase (ALP) activity, total protein (TP) production, alizarin red staining (%) normalized by cell viability (%) obtained by each group/concentration of peptide were submitted to ANOVA/Tukey or *t* Student tests ($p \leq 0.05$), comparing each group of peptide with the control and one each other, at different concentrations. Levels of mRNA gene expression of DSPP and DMP-1 were shown by box-whisker plots and differences among the groups were assessed using Mann-Whitney tests ($p \leq 0.05$). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analyses.

Results

Analysis of cell viability (MTT assays)

The percentage of cell viability obtained by MTT assays after exposure of MDPC-23 odontoblastic-like cells to LL-37, KR-12-a5 and hBD-3-1C^{V} was normalized by the positive control (DMEM, 100% of cell growth). LL-37 and hBD-3-1C^{V} affected minimally cell growth at the concentrations below 62.5 μg/ml. KR-12-a5 was less cytotoxic at the concentrations below 31.25 μg/ml **(Figure 1)**. 0.045% TFA acid solution did not influenced MDPC-23 cell metabolism, promoting 100% of cell growth.

ALP Activity and TP production

The best results were found for LL-37 (62.5 μg/ml), considering ALP activity. hBD-3-1C^{V} at the same concentration had the worst results. Statistical differences were observed for 62.5 μg/ml of hBD-3-1C^{V} compared with the other concentrations of the peptides tested for ALP activity, except when compared with KR-12-a5 at the concentration of 31.25 μg/ml. In relation to TP production, the best results were found to hBD-3-1C^{V} (15.62 μg/ml) compared with the control. The same group was not statistically different from the following groups: hBD-3-1C^{V} (31.25 μg/ml), KR-12-a5 (15.62 and 0.78 μg/ml) and LL-37 (62.5 μg/ml) **(Table 1).**

Mineralized nodule deposition (alizarin red staining)

The percentage of alizarin red staining (%) was normalized by cell viability (%) obtained by each group of peptide, representing mineralized nodule deposition. The highest concentrations of LL-37 and hBD-3-1C^{V} (62.5 μg/ml) and KR-12-a5 (31.25 μg/ml) induced more nodules deposition compared with the control **(Figure 2)**.

Gene expression of odontoblastic markers of dentin mineralization

There were no statistical differences among the groups considering both DSPP and DMP-1 expression **(Figure 3 A and B).**

Discussion

LL-37 and β-defensins belong to a group of small cationic peptides with multiples functions. One of them is the antimicrobial activity against wide-range bacterial, virus and fungi species. Recent discoveries point to important role of these peptides in various cell processes, such as proliferation, migration or chemotaxis (17). However, long length of amino acid chains or chemical linkages difficult their production as therapeutic agent. Then, these peptides are used as template to produce analogues with chemical modifications, but conserving or improving their beneficial properties to human body (12, 14). KR-12 is the smallest region of LL-37 that still maintains the antimicrobial activity of the peptide (13). Analogues of KR-12, such as KR-12-a5, had their antimicrobial activity and LPS-neutralizing improved with minimal chemical changes in the original peptide structure (14). Similar studies were conducted with β-defensins demonstrating that amino acid changes and the absence of disulfide intramolecular

bonds did not affect their antimicrobial activity (12, 26). hBD-3-1C^{v} is an analogue of the β-defensin 3 (hBD-3) which conserved its antibacterial and chemoattractant functions, even replacing 5 of 6 cysteines by alanines and consequently removing their disulfide bridges.

In the present study, LL-37 and hBD-3-1C^{V} affected minimally cell growth at the concentrations below 62.5 μg/ml. Besides, KR-12-a5 was less cytotoxic at concentrations below 31.25 μg/ml. Consuegra et al. (27) demonstrated that LL-37 and KR-12 did not cause toxicity on osteoblasts, erythrocytes and epithelial cells at concentrations less than 100, 50 and 25-50 μg/ml, respectively. The peptide analogue, KR-12, was toxic to macrophages and erythrocytes in the concentrations above 12.5 μM (about 60μg/ml) and 96 μM (about 500μg/ml), respectively. KR-12-a5 was designed by replacing some amino acids like asparagin and glycin by lysines and adding leucins to increase positive charge and hydrophobicity angle. These changes strongly improved the antimicrobial activity and LPS-neutralizing effect of KR-12-a5 in comparison to original LL-37, however, reduced its compatibility to mammalian cells (14). Kluver et al. (28) synthesized hBD-3 analogues and tested antimicrobial activity and cytotoxicity. The authors observed a decreasing on toxicity on monocytic cell lines for hBD-3 analogues compared with the original hBD-3. The analogue of hBD-3, similar to hBD-3-1C^{v}, but with all 6 cysteines replaced by alanines, was toxic at the concentrations above 500μg/ml, ten times less toxic than the original hBD-3 (50μg/ml). The analogue hBD-3-1C^{v} was not toxic for odontoblast-like cells. This is the first study that evaluated cytotoxicity of hBD-3-1C^{v} becoming difficult our comparison with another studies.

Total protein (TP) production is also an indicator of cell metabolism. This study demonstrated a direct relationship between cytotoxity and TP production. KR-12-a5 at the highest concentration tested reduced cell viability and protein production, although statistical difference was not observed when compared with the control. The same was observed for LL-37 at high concentration (8μM) in periodontal ligament cells (11). TP production was reduced to 50% compared with the control and cells reached around 10% of viability (11).

Alkaline phosphatase (ALP) is involved with the initial phase of dentine matrix biomineralization, promoting dephosphorylation of extracellular matrix proteins and providing inorganic phosphate (29). The effect of β-defensins and LL-37 on ALP expression or ALP enzyme activity has been currently studied (17, 18). hBD-2 and hBD-

3 raised ALP enzyme activity significantly in osteoblast-like cells, whereas hBD-1 had no effect (17). The authors demonstrated that hBD-3 positively affected the differentiation of osteoblast-like cells provided by increased transcript levels of osteogenic markers, by upregulated ALP enzyme activity and by enhanced mineralized nodule formation. In this present study, hBD3-1-C^{v}, analogue of hBD3, had no effect on ALP activity, probably because of the modifications on their structure. Higher ALP activity was observed only for LL-37 (62.5 μg/ml) group on MDPC-23 odontoblast-like cells culture, after 7 days in osteogenic medium. Contrarily to our results, Jonsson & Nilsson (11) showed that LL-37 at 0.1 - 8μM had no effect on ALP activity in osteoblast and periodontal ligament cells, for seven days. Zhang & Shively (18) reported that LL-37 induce osteogenic differentiation in monocytes, forming mineralized nodules and expressing intracellular proteins of both osteoblast and osteoclast lineage, except ALP. These data suggested that the ability of LL-37 to stimulate phenotypic markers of mineralization is cell type specific.

After deposition of dentine matrix, mature odontoblasts synthesized proteins, such as dentine matrix phosphoprotein 1(DMP-1), dentine sialoprotein (DSP) and dentine phosphoprotein (DPP) which are expressed during the active mineralization phase of dentinogenesis. DSP and DPP have direct participation in calcium binding to previously synthesized collagenous matrix, initiating the formation of hydroxyapatite crystals within collagen fibres. Both proteins are encoded by the DSPP gene, which is cleaved immediately after secretion (29). DSPP induces organization of intrafibrillar collagen mineralization and DMP-1 stimulates the deposition of mineral particles along the collagen fibril axis (30). In this present study, DMP-1 and DSPP mRNA levels were not affected by 24h peptide exposure and 14 days of osteogenic medium changes. Shiba et al. (16) observed slightly increase of DSPP gene expression after β-defensin 2 (10 μg/ml) exposure in cultures of human pulp-derivated cells and the maximum effect was seen in 6 hours, but not in 24h of exposure at the end of incubation on day 14. Monocytes treated with LL-37 (5μM) for 6 days expressed most proteins related to mineralization produced by osteoblasts, such as BSP II (bone sialoprotein II), using intracellular staining (18).

Although the expression of genes related to mineralization seems not to have been affected by the presence of peptides, mineralized nodules deposition was observed for all groups tested in this study, regardless their concentrations. This result showed

that MDPC-23 cells maintained their odontoblastic phenotype, being able to deposit and mineralize dentine matrix (24). KR-12-a5, hBD-3-1C^{v} and LL-37 at the highest concentration evaluated, increased the alizarin red staining values, showing that these peptides were able to stimulate cells to deposit more quantities of mineralized nodules. Studies were not found evaluating DSPP and DMP-1 gene expression and mineralized nodules deposition after exposure of LL-37, β-defensin 3 or their analogues in odontoblast-like cells. Studies are also necessary to evaluate the expression of other genes related to dentin mineralization or considering different times of peptides exposure.

Conclusion

Although KR-12-a5 had no effect on ALP activity, this peptide analogue stimulated initial mineralized nodules deposition, similar to other peptides, but in lower and minimally cytotoxic concentration. None peptides influenced the expression of DSPP and DMP-1, genes involved in active dentin mineralization.

Acknowledgements

This study was supported by FAPESP (grant # 2013/24606-5), PROPe/CDC-UNESP (grant # 0403/001/14) and CNPq (grant #134551/2013-3).

References

1. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004 Nov 1;15(6):348-81.
2. Byström A, Sundqvist G (1981). Bacteriological evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 89:321-328.
3. Chávez De Paz LE, Dahlén G, Molander A, Möller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J. 2003 Jul;36(7):500-8.
4. Moreno-Hidalgo MC, Caleza-Jimenez C, Mendoza-Mendoza A, Iglesias-Linares A. Revascularization of immature permanent teeth with apical periodontitis. Int Endod J. 2013 Jul 3.

5. Iglesias-Linares A, Yáñez-Vico RM, Sánchez-Borrego E, Moreno-Fernández AM, Solano-Reina E, Mendoza-Mendoza A. Stem cells in current paediatric dentistry practice. Arch Oral Biol. 2013 Mar; 58(3):227-38.
6. Hölzl MA, Hofer J, Steinberge P, Pfistershammer K, Zlabinger GJ. Host anticrobial proteins as endogenous immunomodulators. Immunol Lett. 2008; 119(1-2):4-11.
7. Gorr SU, Aldolhosseini M. Antimicrobial peptides and periodontal disease. J Clin Periodontal. 2011; 38 Suppl 11:126-41.
8. Mccormick TS, Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity Periodontology 2000, 2010; Oct 54(1):195–206.
9. Wiesner J, Vilcinskas A. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 2010 Sep/Oct; 1(5):440-464.
10. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Micros 2003; Dec 36(4); 247-252.
11. Jönsson D, Nilsson BO. The antimicrobial peptide LL-37 is anti-inflammatory and proapoptotic in human periodontal ligament cells. J Periodontal Res. 2012 Jun; 47(3):330-5.
12. Taylor K, Clarke DJ, McCullough B, Chin W, Seo E, Yang D, Oppenheim J, Uhrin D, Govan JR, Campopiano DJ, MacMillan D, Barran P, Dorin JR. Analysis and separation of residues important for chemoattractant and antimicrobial activities of beta-defensin3. J Biol Chem. 2008 Mar 14; 283(11):6631-9.
13. Wang G. Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J Biol Chem. 2008 Nov 21; 283(47):32637-43.
14. Jacob B, Park IS, Bang JK, Shin SY.Short KR-12 analogs designed from human cathelicidin LL37 possessing both antimicrobial and antiendotoxic activities without mammalian cell toxicity. J Pept Sci. 2013 Nov; 19(11):700-7.
15. Kajiya M, Shiba H, Komatsuzawa H, Ouhara K, Fujita T, Takeda K, Uchida Y, Mizuno N, Kawaguchi H, Kurihara H. The antimicrobial peptide LL37 induces the migration of human pulp cells: a possible adjunct for regenerative endodontics. J Endod. 2010 Jun; 36(6):1009-13.
16. Shiba H, Mouri Y, Komatsuzawa H, Ouhara K, Takeda K, Sugai M, Kinane DF, Kurihara H. Macrophage inflammatory protein-3alpha and beta-defensin-2 stimulate dentin

sialophosphoprotein gene expression in human pulp cells. Biochem Biophys Res Commun. 2003 Jul 11; 306(4):867-71.

17. Kraus D, Deschner J, Jäger A, Wenghoefer M, Bayer S, Jepsen S, Allam JP, Novak N, Meyer R, Winter J. Human β-defensins differently affect proliferation, differentiation, and mineralization of osteoblast-like MG63 cells. J Cell Physiol. 2012 Mar; 227(3):994-1003.
18. Zhang Z, Shively JE. Generation of novel bone forming cells (monoosteophils) from the cathelicidin-derived peptide LL-37 treated monocytes. PLoS One. 2010 Nov 15; 5(11):e13985. doi: 10.1371/journal.pone.0013985.
19. Kittaka M, Shiba H, Kajiya M, Fujita T, Iwata T, Rathvisal K, Ouhara K, Takeda K, Fujita T, Komatsuzawa H, Kurihara H. The antimicrobial peptide LL37 promotes bone regeneration in a rat calvarial bone defect. Peptides. 2013 Aug; 46:136-42.
20. Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y. Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. J Periodontal Res. 2007 Oct; 42(5):410-9.
21. Merrifield, R.B., 1963. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide, J. Am. Chem. Soc. 2149–2154.
22. Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34, 595-598.
23. Basso FG, Turrioni AP, Hebling J, de Souza Costa CA. Effects of zoledronic acid on odontoblast-like cells. Arch Oral Biol. 2013 May; 58(5):467-73. doi: 10.1016/j.archoralbio.2012.09.016. Epub 2012 Oct 23.
24. Soares DG, Marcomini N, Basso FG, Pansani TN, Hebling J, de Souza Costa CA. Indirect cytocompatibility of a low-concentration hydrogen peroxide bleaching gel to odontoblast-like cells. Int Endod J. 2014 Dec 30. doi: 10.1111/iej.12426.
25. Ozeki N , Mogi M, Kawai R, Yamaguchi H, Hiyama T, Nakata K, Nakamura H. Mouse-Induced Pluripotent Stem Cells Differentiate into Odontoblast-Like Cells with Induction of Altered Adhesive and Migratory Phenotype of Integrin. PLoS One. 2013 Nov11. 8(11): e80026. Published online 2013; doi: 10.1371.
26. Wu, Z., Hoover, D. M., Yang, D., Boule`gue, C., Santamaria, F., Oppenheim, J. J., Lubkowski, J., and Lu, W. Engineering disulfide bridges to dissect antimicrobial and

chemotactic activities of human â-defensin 3, Proc. Natl. Acad. Sci. U.S.A. 2003; 100, 8880-8885.

27. Consuegra J, de Lima ME, Santos D, Sinisterra RD, Cortés ME. Peptides: β-cyclodextrin inclusion compounds as highly effective antimicrobial and anti-epithelial proliferation agents. J Periodontol. 2013 Dec;84(12):1858-68. doi: 10.1902/jop.2013.120679. Epub 2013 Mar 19.

28. Klüver E, Schulz-Maronde S, Scheid S, Meyer B, Forssmann WG, Adermann K. Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. Biochemistry. 2005; Jul 19;44(28):9804-16.

29. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Frontier of Biosciences. 2011; 3, 711–35.

30. Deshpande AS, Fang PA, Zhang X, Jayaraman T, Sfeir C, Beniash E. Primary Structure and phosphorylation of Dentin Matrix Protein 1 (DMP1) and Dentin Phosphophoryn (DPP) uniquely determine their role in biomineralization. Biomacromolecules. 2011; 12(8):2933–2945.

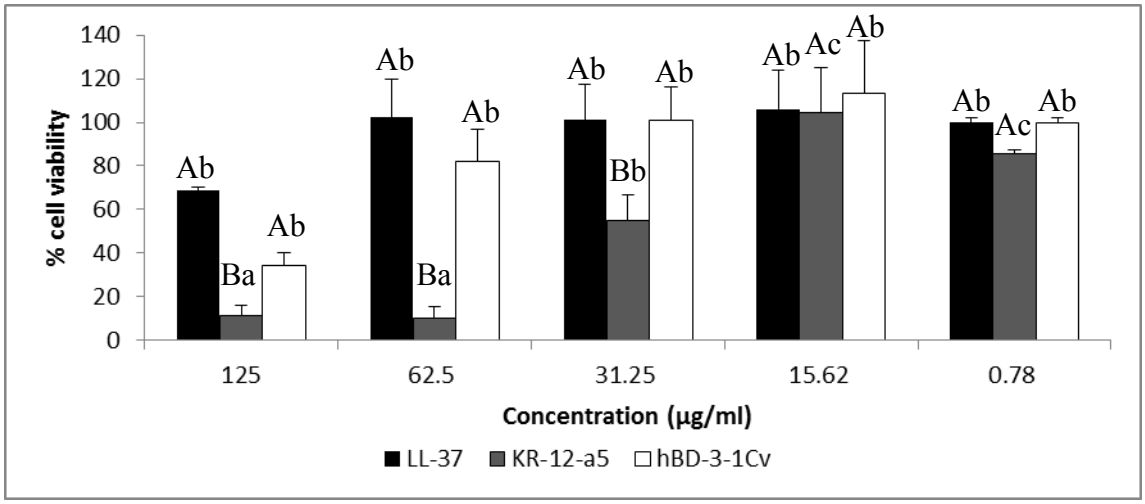


Figure 1. Percentage of MDPC-23 cell viability for each group and concentration in relation to control (DMEM – 100% cell viability) after MTT assays.

[A] Different uppercase letters show statistical differences among the groups, considering each concentration separately, according to ANOVA/Tukey tests ($p \leq 0.05$)
[a] Different lowercase letters show statistical differences among the concentrations, considering each group separately, according to ANOVA/Tukey tests ($p \leq 0.05$)

Table 1. Data from total protein (TP) production and (ALP) alkaline phosphatase activity of MDPC-23 cells exposed to peptides at different concentrations.

Peptide	Concentration (µg/ml)	TP production (U/l)	ALP activity (U/l)
LL-37	62.5	82.31 (11.25)^{A,b}	55.09 (6.28)^{A,a}
	31.25	79.07 (14.55)^{A,a}	49. 18 (3.48)$^{B,a/b}$
	15.62	73.68 (10.39)^{A,a}	47.28 (4.98)$^{B,a/b}$
KR-12-a5	31.25	68.42 (20.92)^{A,a}	40.71 (4.2)$^{B,b/c}$
	15.62	81.18 (34.11)^{A,b}	49.25 (5.43)$^{B,a/b}$
	0.78	80.64 (20.62)^{A,b}	48.32 (5.62)$^{B,a/b}$
hBD-3-1C^{v}	62.5	77.59 (19.94)^{A,a}	31.9 (2.72)^{A,c}
	31.25	101.93 (26.75)^{A,b}	43.52 (5.46)^{B,b}
	15.62	120.14 (26.50)^{B,b}	45.45 (7.51)$^{B,a/b}$
Control	Osteogenic médium	74.34 (16,08)^{A}	45.21 (4.77)^{B}

^{A} Different uppercase letters show statistical differences between each group of peptide and control, according to *t* Student tests ($p \leq 0.05$).
^{a} Different lowercase letters show statistical differences among the concentrations of peptides, according to ANOVA/Tukey tests ($p \leq 0.05$).

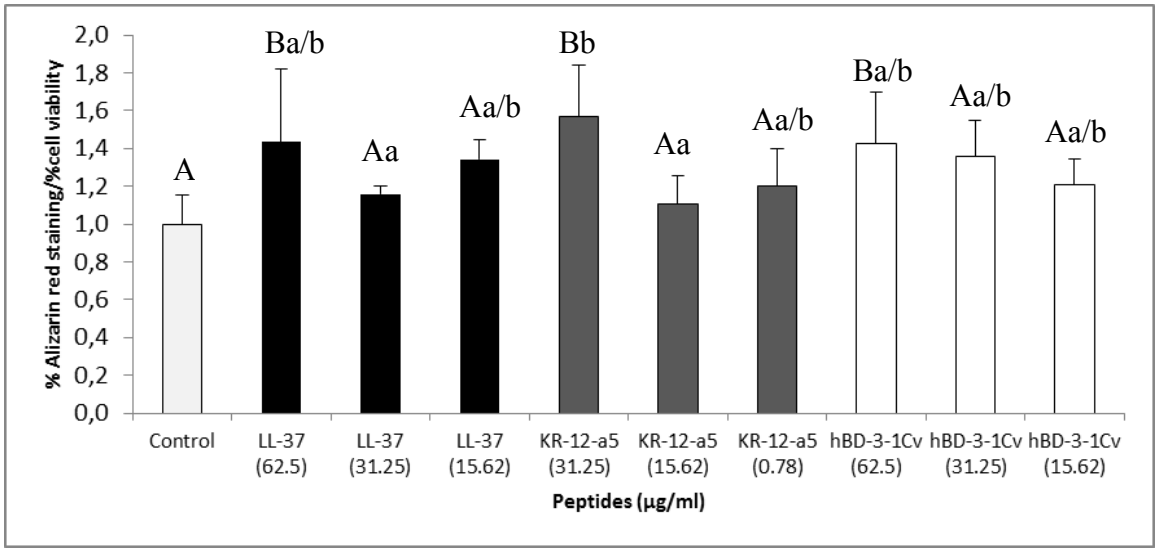


Figure 2. Percentage of alizarin red staining (%) normalized by viability cell results obtained by each group of peptide, representing mineralized nodule deposition.

[A] Different uppercase letters show statistical differences between each group of peptide and control, according to ANOVA/Tukey tests ($p \leq 0.05$)
[a] Different lowercase letters show statistical differences among the concentrations of peptides, according to ANOVA/Tukey tests ($p \leq 0.05$).

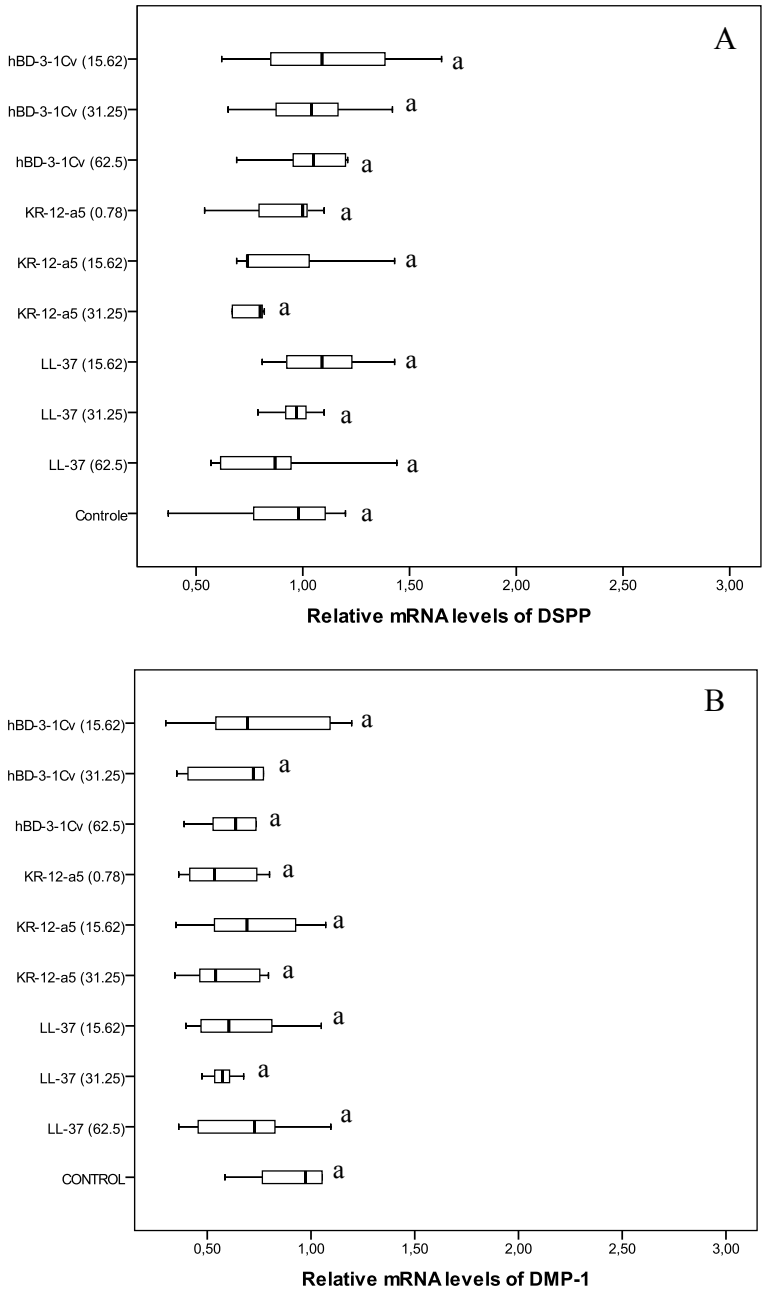


Figure 3. Box-whisker plots of mRNA gene expression of DSPP (A) and DMP-1 (B). Vertical axis represents the experimental groups and horizontal axis represents relative gene expression normalized by the negative control group (gene GAPDH).

[a] Different lowercase letters show statistical differences among the groups, according to Mann-Whitney tests ($p \leq 0.05$).

Anexos

ANEXO A

Guide for Authors (Journal of Endodontics)

Introduction

The *Journal of Endodontics* is owned by the American Association of Endodontists. Submitted manuscripts must pertain to endodontics and may be original research (eg, clinical trails, basic science related to the biological aspects of endodontics, basic science related to endodontic techniques, case reports, or review articles related to the scientific or applied aspects of endodontics). Clinical studies using CONSORT methods (http://www.consort-statement.org/consort-statement/) or systematic reviews using meta-analyses are particularly encouraged. Authors of potential review articles are encouraged to first contact the Editor during their preliminary development via e-mail at *JEndodontics@UTHSCSA.edu*. Manuscripts submitted for publication must be submitted solely to *JOE*. They must not be submitted for consideration elsewhere or be published elsewhere.

Disclaimer

The statements, opinions, and advertisements in the *Journal of Endodontics* are solely those of the individual authors, contributors, editors, or advertisers, as indicated. Those statements, opinions, and advertisements do not affect any endorsement by the American Association of Endodontists or its agents, authors, contributors, editors, or advertisers, or the publisher. Unless otherwise specified, the American Association of Endodontists and the publisher disclaim any and all responsibility or liability for such material.

Before You Begin

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethicsand http://www.elsevier.com/journal-authors/ethics.

Human and animal rights

If the work involves the use of animal or human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/en/30publications/10policies/b3/index.html; EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm;

Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

Conflict of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this: 'Conflicts of interest: none'. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/sharingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts: *Before the accepted manuscript is published in an online issue*: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Reporting clinical trials

Randomized controlled trials should be presented according to the CONSORT guidelines. At manuscript submission, authors must provide the CONSORT checklist accompanied by a flow diagram that illustrates the progress of patients through the trial, including recruitment, enrollment, randomization, withdrawal and completion, and a detailed description of the randomization procedure. The CONSORT checklist and template flow diagram can be found on http://www.consort-statement.org.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions. For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted third party reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. For more information see http://www.elsevier.com/copyright.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report;

and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some authors may also be reimbursed for associated publication fees. To learn more about existing agreements please visit http://www.elsevier.com/fundingbodies.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submit your article

Please submit your article via http://ees.elsevier.com/joe/.

Preparation

General Points on Composition

Authors are strongly encouraged to analyze their final draft with both software (eg, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery because their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision or that have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the Journal of Endodontics: a. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph

provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section "c," below), or sentences with little to no transition within a paragraph.

b. Keep to the point. The subject of the sentence should support the subject of the paragraph For example, the introduction of authors' names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, "In 1983, Langeland et al, reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals" can be edited to: "Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al, 1983)." In this example, the paragraph's subject is sodium hypochlorite and sentences should focus on this subject.

c. Sentences are stronger when written in the active voice, that is, the subject performs the action. Passive sentences are identified by the use of passive verbs such as "was," "were," "could," etc. For example: "Dexamethasone was found in this study to be a factor that was associated with reduced inflammation," can be edited to: "Our results demonstrated that dexamethasone reduced inflammation." Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

d. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus, or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.) e. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related. f. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study." g. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

h. Authors should be aware that the JOE uses iThenticate, plagiarism detection software, to

ensure originality and integrity of material published in the journal. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by the new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the

work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Structured abstract

A structured abstract, by means of appropriate headings, should provide the context or background for the research and should state its purpose, basic procedures (selection of study subjects or laboratory animals, observational and analytical methods), main findings (giving specific effect sizes and their statistical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations.

Abstract Headings

Introduction, Methods, Results, Conclusions

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

The authors deny any conflicts of interest related to this study.

Original Research Article Guidelines

Title Page

The title describes the major emphasis of the paper. It must be as short as possible without loss of clarity. Avoid abbreviations in the title because this may lead to imprecise coding by electronic citation programs such as PubMed (eg, use sodium hypochlorite rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at www.icmje.org). Include the manuscript title; the names and affiliations of all authors; and the name, affiliation, and full mailing address (including e-mail) of the corresponding author. This author will be responsible for proofreading page proofs and ordering reprints when applicable. Also highlight the contribution of each author in the cover letter.

Abstract

The Abstract concisely describes the purpose of the study in 250 or fewer words. It must be organized into sections: Introduction, Methods, Results, and Conclusions. The hypothesis is described in the Abstract Introduction. The Abstract describes the new contributions made by

this study. The Abstract word limitation and its wide distribution (eg, PubMed) make it challenging to write clearly. This section is written last by many authors. Write the abstract in past tense because the study has been completed. Provide 3-5 keywords.

Introduction

The introduction briefly reviews the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. Clearly describe the purpose of the study, the tested hypothesis, and its scope. Many successful manuscripts require no more than a few paragraphs to accomplish these goals; therefore, do not perform extensive literature review or discuss the results of the study in this section.

Materials and Methods

The Materials and Methods section is intended to permit other investigators to repeat your experiments. There are 4 components to this section: (1) detailed description of the materials used and their components, (2) experimental design, (3) procedures employed, and (4) statistical tests used to analyze the results. Most manuscripts should cite prior studies that used similar methods and succinctly describe the essential aspects used in the present study. A "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then you must carefully describe the method and include validation experiments. If the study used a commercial product, the manuscript must either state that you followed manufacturer's protocol or specify any changes made to the protocol. If the study used an *in vitro* model to simulate a clinical outcome, describe either experiments made to validate the model or previous literature that proved the clinical relevance of the model. The statistical analysis section must describe which tests were used to analyze which dependent measures; *P* values must be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, dropouts from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

Results

Only experimental results are appropriate in this section; do not include methods, discussion, or conclusions. Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be rejected from publication. All Figures, Charts, and Tables must be cited in the text in numerical order and include a brief description of the major findings. Consider using Supplemental Figures, Tables, or Video clips that will be published online. Supplemental material often is used to provide additional information or control experiments that support the results section (eg, microarray data).

Figures

There are 2 general types of figures: type 1 includes photographs, radiographs, or micrographs; type 2 includes graphs. *Type 1:*Include only essential figures and use composite figures containing several panels of photographs, if possible. Each panel must be clearly identified with a letter (eg, A, B, C), and the parts must be defined in the figure legend. A figure that contains many panels counts as 1 figure. *Type 2:* Graphs (ie, line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). One example is a graph depicting pain scores over time. Use graphs when the overall trend of the results is more important than the exact numeric values of the results. A graph is a convenient way to report that an ibuprofen-treated group reported less pain than a placebo-treated group over the first 24 hours, but pain reported was the same for both groups over the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

Tables

Tables are appropriate when it is critical to present exact numeric values; however, not all results need be placed in either a table or figure. Instead of a simple table, the results could state that there was no inhibition of growth from 0.001%-0.03% NaOCl, and a 100% inhibition of growth from 0.03%-3% NaOCl (N=5/group). If the results are not significant, then it is probably not necessary to include the results in either a table or as a figure.

Acknowledgments

All authors must affirm that they have no financial affiliation (eg, employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements, or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past 3 years. Disclose any potential conflict of interest. Append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Disclose all sources and attribute all grants, contracts, or donations that funded the study. Specific wording: "The authors deny any conflicts of interest related to this study."

References

The reference style can be learned from reading past issues of *JOE.* References are numbered in order of citation. Place text citation of the reference Arabic number in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

Other Article Types and Guidelines

Manuscripts submitted to *JOE* that are not Original Articles must fall into one of the following categories. Abstract limit: 250 words. Note that word limits, listed by type, do not include figure legends or References. If you are not sure whether your manuscript falls within one of the categories listed or if you would like to request pre-approval to submit additional figures, contact the Editor at*JEndodontics@uthscsa.edu*.

CONSORT Randomized Clinical Trial

Must strictly adhere to the Consolidated Standards of Reporting Trials—CONSORT—minimum guidelines for publication of randomized clinical trials (http://www.consort-statement.org). Word limit: 3500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables:

4. *Review Article*

Either narrative articles or systemic reviews/meta-analyses. Case Report/Clinical Techniques articles, even when they include an extensive review of the literature, are categorized as Case Report/Clinical Techniques. Word limit: 3500. Headings: Abstract, Introduction, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables:

4. *Clinical Research*

Prospective or retrospective studies of patients or patient records, research on biopsies excluding the use of human teeth for technique studies. Word limit: 3500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables:

4. *Basic Research—Biology*

Animal or culture studies of biological research on physiology, development, stem cell differentiation, inflammation, or pathology. Primary focus is on biology. Word limit: 2500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables:

4. *Basic Research—Technology*

Focus primarily on research related to techniques and materials used, or on potential clinical use, in endodontics. Word limit: 2500. Headings: Abstract, Introduction, Material and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 3. Maximum number of tables:

3. *Case Report/Clinical Techniques*

Reports of an unusual clinical case or use of a cutting edge technology in a clinical case. Word limit: 2500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4. **Units**

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures

will appear in color online (e.g., ScienceDirect and other sites) in addition to color reproduction in print. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not allowed in the reference list, but they may be mentioned in the text. Citation of a reference as "in press" implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web References

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references are included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have a standard template available in key reference management packages. This covers packages using the Citation Style Language, such as Mendeley (

http://www.mendeley.com/features/reference-manager) and also others like EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to word processing packages which are available from the above sites, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style as described in this Guide. The process of including templates in these packages is constantly ongoing. If the journal you are looking for does not have a template available yet, please see the list of sample references and citations provided in this Guide to help you format these according to the journal style.

If you manage your research with Mendeley Desktop, you can easily install the reference style for this journal by clicking the link below:

http://open.mendeley.com/use-citation-style/journal-of-endodontics

When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice. For more information about the Citation Style Language, visit http://citationstyles.org.

Reference style

Text: Indicate references by Arabic numerals in parentheses, numbered in the order in which they appear in the text. *List:* Number the references in the list in the order in which they appear in the text. List 3 authors then et al.

Examples:

Journal article:

Van der Geer J, Hanraads JAJ, Lupton RA. Thert of writing a scientific article. J Sci Commun. 2010;163:51–59.Book:

2. Strunk W Jr, White EB. *The Elements of Style,* 4th ed. New York: Longman; 2000. Chapter in an edited book:

3. Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, eds. *Introduction to the Electronic Age.* New York: E-Publishing; 2009:281–304.

Journal abbreviations source

Journal names are abbreviated according to Index medicus.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation

material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Audio Slides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary material

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Database linking

Elsevier encourages authors to connect articles with external databases, giving readers access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported

databases.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)

Printed version of figures (if applicable) in color or black-and-white

• Indicate clearly whether or not color or black-and-white in print is required.

• For reproduction in black-and-white, please supply black-and-white versions of the figures for printing purposes.

ANEXO B

Análise da Síntese de LL-37

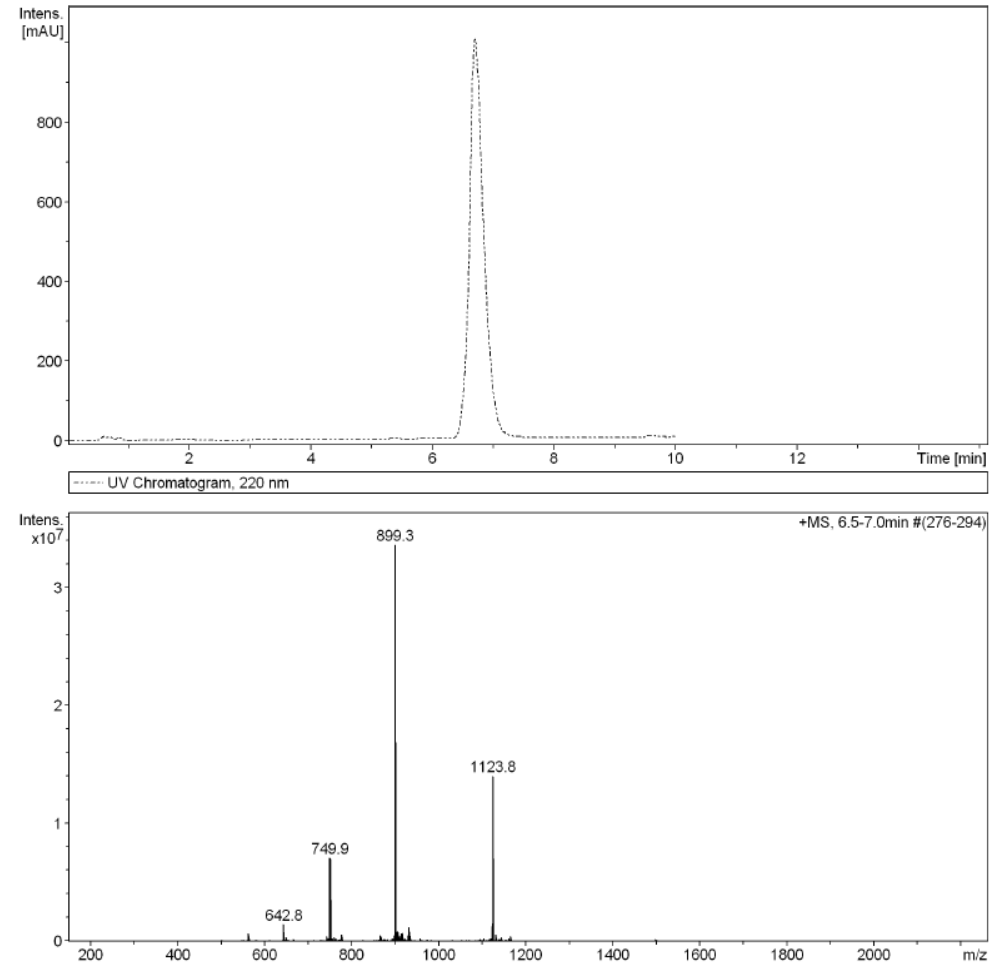
Display Report

Analysis Info

Analysis Name	D:\Data\Norival\2014\NA1350_1-2_01_1811.d	Acquisition Date	9/26/2014 12:10:40 PM
Method	1811.m	Operator	emcilli
Sample Name	NA1350	Instrument	amaZon SL
Comment			

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Enhanced	Scan Begin	70 m/z	Scan End	2200 m/z
Capillary Exit	Resolution	n/a	n/a	Trap Drive	76.9
Accumulation Time	100 µs	Averages	5 Spectra	Auto MS/MS	off



Bruker Compass DataAnalysis 4.0 printed: 7/4/2015 4:53:20 PM Page 1 of 1

Análise da Síntese de KR-12-a5

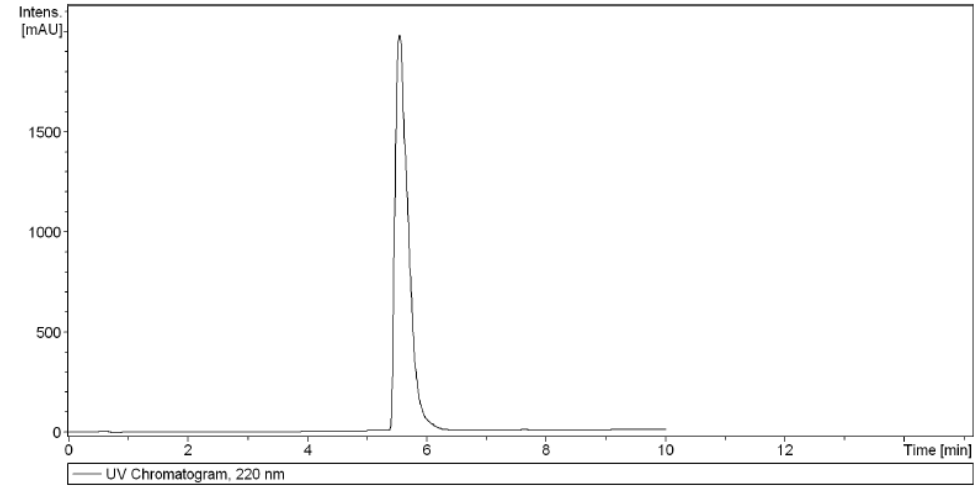
Display Report

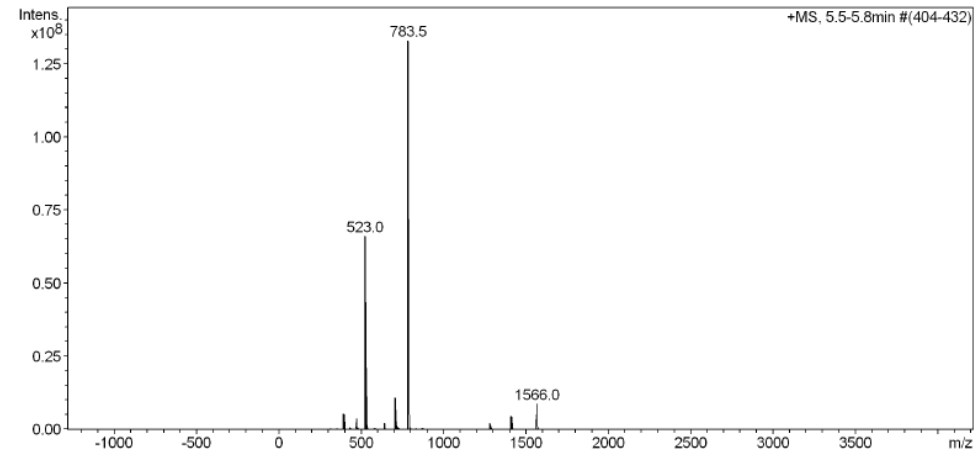
Analysis Info

Analysis Name	D:\Data\Norival\NA1349_2ndPur_270314_1-3_01_1307.d	Acquisition Date	3/28/2014 11:26:37 AM
Method	1307.m	Operator	emcilli
Sample Name	NA1349_2ndPur_270314	Instrument	amaZon SL
Comment			

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Extended Mass	Scan Begin	200 m/z	Scan End	2200 m/z
Capillary Exit	[illegible]	n/a	n/a	Trap Drive	76.9
Accumulation Time	10353 µs	Averages	8 Spectra	Auto MS/MS	off





Bruker Compass DataAnalysis 4.0 printed: 7/4/2015 4:28:23 PM Page 1 of 1

Análise da Síntese de hBD-3-1C^{V}

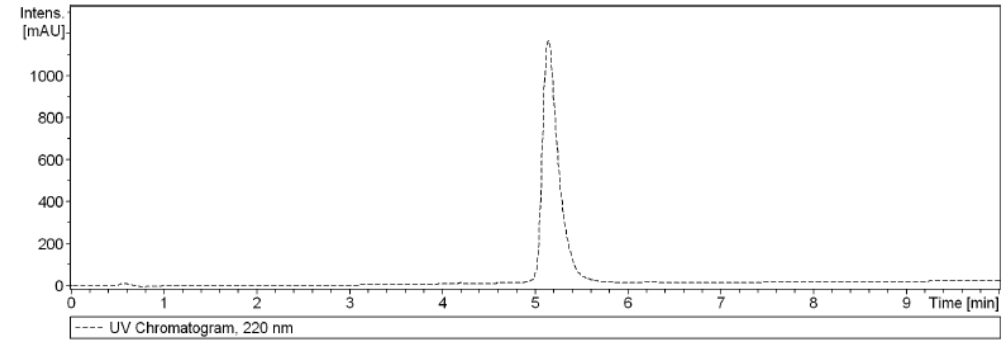
Display Report

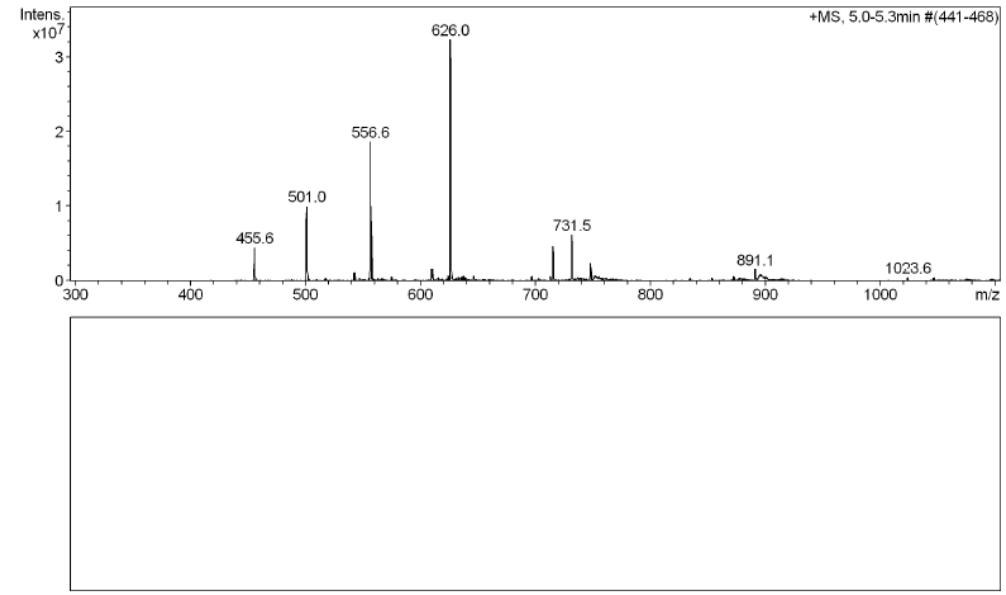
Analysis Info

Analysis Name	D:\Data\Norival\NA1366-280314\NA1366-tb8a10_1-3_01_1329.d	Acquisition Date	4/1/2014 4:54:48 PM
Method	1329.m	Operator	emcilli
Sample Name	Na1366-Tb8a10	Instrument	amaZon SL
Comment			

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	UltraScan	Scan Begin	200 m/z	Scan End	2200 m/z
Capillary Exit	140.0 V	n/a	n/a	Trap Drive	68.8
Accumulation Time	4390 µs	Averages	8 Spectra	Auto MS/MS	off





Bruker Compass DataAnalysis 4.0 printed: 7/4/2015 4:49:29 PM Page 1 of 1

ANEXO C

PROTOCOLO DA COLORAÇÃO DE GRAM

1. Limpar rigorosamente a lâmina e identificar;
2. Fazer um esfregaço bem homogênio, a partir de uma cultura em caldo, fixar pelo calor (bico de Bunsn por 3 vezes) e esperar esfriar;
3. Cobrir a lâmina com cristal violeta e deixar agir por 1 minuto;
4. Lavar rapidamente em água corrente (fio);
5. Tratar com solução de lugol por 1 minuto;
6. Lavar em água corrente e secar bem;
7. Descorar com álcool acetona (aproximadamente 30 segundos), com ligeira agitação;
8. Lavar em água corrente;
9. Tratar com solução de Fucsina ou Safranina por 10 segundos;
10. Lavar em água, deixar secar e observar na objetiva desejada.

Anexo D

Protocolo de Determinação da MIC e MLC

1º dia experimental: Proceder com a reativação do microrganismo (MO) em Brain Heart Infusion (BHI) agar para as bactérias facultativas, Sabouraud Dextrose (SD) agar para *C. albicans* e BHI sangue para bactérias anaeróbias estritas (estriar 15µl da cultura estoque em Técnica de esgotamento) e incubar de acordo com as exigências de ambiente requeridas para cada microrganismo e experimento.

2º dia experimental: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 a 10 UFC em 5ml) e incubar.

3º dia experimental: Diluição dos antimicrobianos teste:

→ Pipetar 50µl do peptídeo e controle de Clorexidina, ressuspendidos na concentração inicial que se deseja (mg/ml) no primeiro e segundo poço de uma placa de 96 poços.

→ Pipetar 50µl de água deionizada estéril em cada poço, exceto no primeiro.

→ Ressuspender o segundo poço e transferir 50µl para o terceiro poço, e assim sucessivamente (Técnica de diluição seriada).

* Preparar o inoculo de acordo com a curva de crescimento: D.O. 0,5 para as bactérias facultativas, 0,3 para *C. albicans e* para *F. nucleatum* e D.O. 0,2 para *P. gingivalis*.

* Mueller-Hinton caldo para as bactérias anaeróbias facultativas, RPMI-1640 para *C. albicans* e BHI caldo suplementado com Hemina e Menadiona para as bactérias anaeróbias estritas.

→ Diluir 1000x no meio de cultura 2x concentrado (500µl do inoculo em 4,5ml de meio).

→ Ressuspender 50µl do inoculo em cada poço; tampar a placa e incubar por 24h em condições específicas para cada microrganismo.

4º dia experimental: Pipetar 15µl de solução de Resazurina 0,01% em cada poço e incubar por 4h.

→ Plaquear o último poço com coloração azulada (CIM) e, no mínimo, 2 poços anteriores para se determinar a CLM (Diluição de 10 para 90 µl/ 0-7 quadrantes).

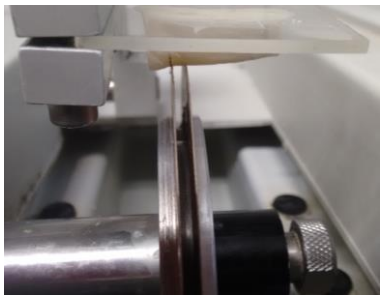
5º dia experimental: Leitura das placas em absorbância de 550 a 600 nm (dependo do MO analizado) e plaqueamento / contagem das UFC/ml.

ANEXO E

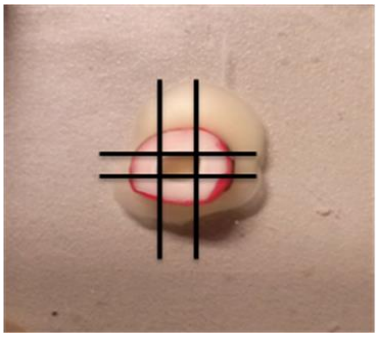
OBTENÇÃO E PREPARO DOS BLOCOS DE DENTINA RADICULAR

Confecção dos blocos de dentina radicular bovino (3 mm x 3 mm)

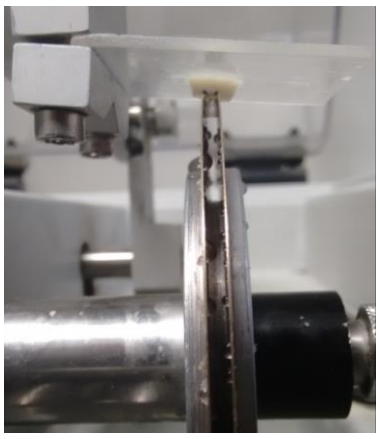
1. Raízes de dente bovino (incisivo central), separada da coroa através de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob-refrigeração (água destilada/deionizada), após o corte, marcação de 1mm abaixo da junção amelocementária.



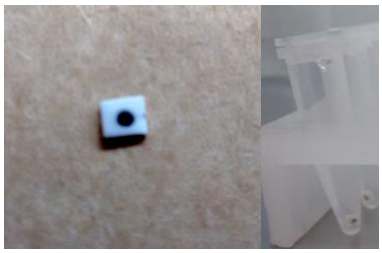
2. Secção da porção radicular utilizando disco diamantado (Extec Diamond Wafer Blade, série 12205,102mm X 0,3mm X 12,7mm, Extec Corporation, Enfield, CT, USA).



3. Disco da porção radicular fixada no acrílico e com as marcações de corte para obtenção dos quatro blocos de dentina radicular (3mm X 3mm X 0,7mm).



4. Secção dos quatro blocos no sentido longitudinal, respeitando marcações prévias, utilizando-se de 2 discos (Extec Diamond Wafer Blade, série 12205,102mm X 0,3mm X 12,7mm, Extec Corporation, Enfield, CT, USA)., montados em cortadeira (ISOMET 1000 PRECISION SAW – BUEHLER) sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 3 mm de espessura. Em seguida, foi realizado o corte no sentido transversal.



5. Fragmento de dentina radicular do dente bovino, com marcação da porção de cemento e ao lado, o bloco em eppendorf com gaze umedecida em formol a 2%.

Planificação do cemento e polimento da dentina

6. Bloco de dentina fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 8 mm de espessura), com auxílio de cera pegajosa (Kota Ind. e Com. LTDA), com a superfície cementária voltada para cima.

7. Ajuste do cemento para obtenção de superfícies paralelas entre dentina e cemento, utilizando Politriz VECTOR POWER HEAD BUEHLER e lixas de granulação 320 (CARBIMET Paper Disks, 30-5108-320, BUEHLER), durante ± 7 segundos, dependendo da espessura inicial, sob-baixa rotação e refrigeração.

8. Para polimento da dentina radicular profunda, a fim de remover o mínimo possível, utilização de lixas de 400 e 600 de forma manual e polimento em Politriz com lixa de granulação 1200 (CARBIMET Paper Disks, BUEHLER), durante ± 5 segundos, dependendo da espessura inicial, sob-baixa rotação e refrigeração, a espessura final para os testes de Biofilme é ± 0,7mm e para o Confocal de ± 0,5mm. E descontaminação com EDTA 17% em ultrassom por 3 min. e 5min. com H2O deionizada e esterilização em autoclave.

Sequencia do polimento e descontaminação da dentina radicular

1. A planificação inicial deve ser feita no cemento, utilizando Politriz VECTOR POWER HEAD BUEHLER e lixas de granulação 320 (CARBIMET Paper Discs, 30-5108-320, BUEHLER), durante ± 7 segundos, dependendo da espessura inicial, sob-baixa rotação e refrigeração.
2. A planificação e o polimento da dentina profunda devem ser realizados cuidadosamente a fim de não desgasta-la excessivamente, utilizando lixas de 400 e 600 de forma manual e polimento em Politriz com lixa de granulação 1200 (CARBIMET Paper Discs, BUEHLER), durante ± 5 segundos, dependendo da espessura inicial, sob-baixa rotação e refrigeração, a espessura final para os testes de Biofilme é ± 0,7mm e para o Confocal de ± 0,5mm.
3. Para remoção de *smear layer* utiliza-se de EDTA a 17% em lavadora ultrassônica por 3 minutos.
4. Limpeza em lavadora ultrassônica utilizando água destilada/deionizada por 5 minutos a fim de remover resquícios de *smear layer* e EDTA;
5. Após lavagem, a parte de cemento do bloco recebe uma fita de dupla face e em seguida é esterilizados em pacote de grau cirúrgico em autoclave por 15 minutos a 121ºC.

   OBS 1: A fita dupla face serve para prender o bloco de dentina no fundo dos poços das placas com a porção de dentina profunda voltada para cima em contato com o meio e os materiais testados.

   OBS 2: O cuidado que se teve com os quatro blocos do mesmo dente, foi aleatorizá-los, para que eles não pudessem ficar nos mesmos grupos experimentais.

ANEXO F

PROTOCOLO DO ENSAIO DE BIOFILME

1. Protocolo de Biofilme Formado

(Concentrações dos peptídeos e do controle de Clorexidina são correspondentes à 5x e 10x a Concentração Bactericida Mínima)

→ Coletar uma alçada de microrganismos (5 – 10 UFC) para um tubo contendo 5 ml de BHI caldo (quando para MO anaeróbios suplementados com Hemina e Menadiona) com glicose (0,5% para *E.faecalis* e 1% para *F. nucleatum*) e incubar por 24 horas em condições favoráveis de crescimento para cada MO testado.

→ Para crescimento da D.O., após 24horas de crescimento, coletar uma alíquota de 50µl da cultura de *E. faecalis* e 1 ml da cultura de *F. nucleatum*, e acrescentar em tubos contendo 5 ml de BHI caldo com glicose suplementados ou não dependendo do MO, incubar por 3horas e 30 min. em CO2 a 37ºC (*E.faecalis*) e 5 horas em estufa de anaerobiose a 37ºC (*F. nucleatum*).

→Realizar uma diluição de 1000x em 4,5 ml de BHI caldo (supl.) com glicose em concentração normal (1x concentrado).

Montagem da placa com os blocos de dentina:

*Os blocos de dentina esterilizados (3mm X 3mm X 0,7mm) devem ser manipulados com auxílio de pinças estéreis, e uma parte da película da fita dupla face utilizada para fixar os bloquinhos no fundo dos poços deve ser removida e o bloco fixado da placa de 96 poços, a porção de dentina radicular profunda deve ficar em contato com o meio.

→ Acrescentar 100µl da cultura diluída em cada poço, sendo dois poços usados como controles (um com BHI 1X estéril e outro com cultura).

→ Incubar por 48 horas tanto para *E. faecalis* quanto para *F.nucleatum*.

→ Retirar a solução dos poços e lavar uma vez com 100µl de salina 0,9%.

→Acrescentar aos poços a concentração exata dos antimicrobianos, sendo dois poços usados como controles (um com BHI 1X puro e outro com cultura) nesses, o BHI devem ser removidos e acrescentados 100µl de água deionizada estéril.

→Incubar por 24 horas.

→Após incubar, retirar a solução dos poços e lavar duas vezes com 100µl de salina 0,9%.

→Com auxílio de pinças, retirar os blocos de dentina dos poços e remover a fita dupla face.

→ Inserir cada bloco em eppendorf contendo 500µl de salina 0,9%.

Protocolo de aquisição de microrganismos em blocos de dentina:

- ➔ Colocar os eppendorfs contendo os blocos por 20 min. em ultrassom;
- ➔ Vortexar por 30 segundos;
- ➔ Sonicar por 30 segundos em 30 Watts;
- ➔ Vortexar por mais 1 minuto.

→ Realizar a diluição de 50µl (do eppendorf de 500µl com as bactérias soltadas) para 450µl de salina e assim sucessivamente.

→Fazer plaqueamento em BHI Agar para *E. faecalis* e BHI sangue para *F. nucleatum*, em cada quadrante pipetar 2 gotas de 25µl.

→Incubar em condições favoráveis para cada MO estudado e realizar a contagem das UFC/ml.

ANEXO G

MICROSCOPIA CONFOCAL

LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152)

Programa: LAS AF Leica Microsystems

Análise das imagens por Image J

(Concentrações dos peptídeos e do controle de Clorexidina
são correspondentes à 5x a Concentração Bactericida Mínima)

1. Coletar uma alçada de microrganismos (5 – 10 UFC) para um tubo contendo 5 ml de BHI caldo com glicose (0,5% para *E. faecalis*) e incubar por 24 horas em condições favoráveis de crescimento para cada MO testado;
2. Para crescimento da D.O., após 24horas de crescimento, coletar uma alíquota de 50µl da cultura de *E. faecalis* e acrescentar em tubos contendo 5 ml de BHI caldo com glicose, incubar por 3horas e 30 min. em CO2 a 37ºC (*E. faecalis*);
3. Realizar uma diluição de 1000x em 4,5 ml de BHI caldo (supl.) com glicose concentrado;

Montagem da placa com os blocos de dentina:

*Os blocos de dentina esterilizados (3mm X 3mm X 0,5mm) devem ser manipulados com auxílio de pinças estéreis, e uma parte da película da fita dupla face utilizada para fixar os bloquinhos no fundo dos poços deve ser removida e o bloco fixado da placa de 96 poços, a porção de dentina radicular profunda deve ficar em contato com o meio;

4. Acrescentar 100µl da cultura diluída em cada poço;
5. Três poços devem ser usados como controles (Apenas com cultura);
6. Incubar por 24 horas;
7. Retirar a solução dos poços;
8. Acrescentar aos poços 5X a concentração de MBC dos antimicrobianos, e água deionizada estéril nos poços controles de cultura;
9. Incubar por 4 horas;

10. Após incubar, retirar a solução dos poços e lavar com 100µl de água deionizada estéril;
11. Aplicar 100µl da solução do Kit Live/Dead (contendo SYTO 9 e Iodo propídeo) em cada poço (solução confeccionada de acordo com as instruções do fabricante);
12. Incubar por 15 minutos no escuro;
13. Com auxílio de pinças, retirar os blocos de dentina dos poços e remover a fita dupla face, secando levemente os blocos de dentina com lenço de papel macio;
14. Montar o bloco de dentina sobre a lamínula do microscópio (Leica TCS SP5, Microsystems GmbH) utilizando o óleo do Kit, certificando-se que a parte que se quer analisar esteja para baixo;
15. Comprimentos de onda de excitação e emissão: 480/500 nm para SYTO 9 e 490/635 nm para Iodo propídeo**;**
16. A fluorescência é captada pelo equipamento Leica TCS SP5, Microsystems GmbH;
17. Objetiva de 63X (lente que deve ser usada com óleo de imersão);
18. Software LAS AF Leica Microsystems com resolução de 1024 por 1024 pixels por imagem;

OBS. Devem ser selecionadas mais de uma área para captação das imagens, sendo uma delas utilizada para realizar as imagens tridimensionais através da função Z step size;

19. A quantificação das bactérias vivas e mortas é realizada pelo programa image J 1,48.

ANEXO H

ENSAIO DE MTT (L929) – Análise de Viabilidade celular (24 HORAS)

1. Realizar plantio das células (Fibroblasto L929) semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços (100µl → 7 X 10^5 por poço);
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 24 horas para análise da viabilidade celular.
6. Após incubação, aspirar o meio e acrescentar a 100µl solução de MTT (100µl da solução de MTT em uma concentração de 5mg/ml em PBS 1X e 900 µl de DMEM sem SFB)
7. Incubar por 4 horas;
8. Aspirar a solução de MTT;
9. Acrescentar 100µl em cada poço de solução de Isopropanol acidulada;
10. Homogeneizar;
11. Leitura da absorbância em 570 nm em espectrofotômetro.

ANEXO I

ENSAIO DE MTT (MDPC-23) – Análise de Viabilidade celular

(24 HORAS E 7 DIAS)

1. Realizar plantio das células semelhantes à odontoblastos MDPC – 23 semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços (100µl → 1 X 10^4 por poço);
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 24 horas para análise da viabilidade celular e outra placa por 7 dias como controle de viabilidade dos demais testes (Fosfatase Alcalina, Proteína Total e Alizarin – Coloração de nódulos de mineralização).

   OBS. Quando o ensaio de MTT durar 7 dias, o meio deve ser trocado a cada 24 horas pelo meio osteogênico que induz a diferenciação celular.

6. Após incubação, aspirar o meio e acrescentar a 100µl solução de MTT (100µl da solução de MTT em uma concentração de 5mg/ml em PBS 1X e 900 µl de DMEM sem SFB)
7. Incubar por 4 horas;
8. Aspirar a solução de MTT;
9. Acrescentar 100µl em cada poço de solução de Isopropanol acidulada;
10. Homogeneizar;
11. Leitura da absorbância em 570 nm em espectrofotômetro.

ANEXO J

ENSAIO DE PRODUÇÃO DE PROTEÍNA TOTAL (7 DIAS)

1. Após realizar o plantio das células semelhantes à odontoblastos MDPC – 23 semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços;
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 7 dias realizando trocas do meio a cada 24 horas por meio osteogênico (meio de diferenciação) – DMEM com 10% SFB, suplementado com 50 μg/mL $^{-1}$ de ácido ascórbico e 10 nmol L $^{-1}$ β-glicerofosfato; Sigma/Aldrich Corp;
6. Após o período de 7 dias, aspirar o meio;
7. Pipetar 150μl de detergente Lauryl Sulfato de Sódio a 0,1% (Lise celular);
8. Incubar em temperatura ambiente por 40 minutos;
   OBS: Desses 150μl: 100μl foram usados para o ensaio de PT e 50μl para a Fosfatase Alcalina.
9. Acrescentar 100μl em cada poço do reagente de Lowry (vai se ligar as proteínas dissolvidas no meio e gerar alteração do pH);
10. Incubar por 20 minutos em temperatura ambiente;
11. Pipetar em cada amostra 50μl do reagente Folin (fotossensível);
12. Incubar por 30 minutos em temperatura ambiente no escuro;
13. Realizar leitura em 655nm.

OBS: Uma Curva de Proteína Padrão, com concentrações já conhecidas, é realizada da seguinte forma:

1º Ponto da curva: 100μl de Lauril;

2º Ponto da curva: 80μl de Lauril + 20μl de BSA (Albumina Bovina) – [32 μg/ml];

3º Ponto da curva: 60µl de Lauril + 40µl de BSA (Albumina Bovina) – [64 µg/ml];

4º Ponto da curva: 40µl de Lauril + 60µl de BSA (Albumina Bovina) – [96 µg/ml];

5º Ponto da curva: 20µl de Lauril + 80µl de BSA (Albumina Bovina) – [128 µg/ml];

6º Ponto da curva: 100µl de BSA (Albumina Bovina) – [160 µg/ml]

ANEXO K

ENSAIO DA ATIVIDADE DE FOSFATASE ALCALINA (7 DIAS)

OBS: Protocolo utilizado de acordo com o fabricante (ALP Kit, reference 40; Lot. 4001; Labtest Diagnóstico S.A., Lagoa Santa, MG, Brasil)

1. Após realizar o plantio das células semelhantes à odontoblastos MDPC – 23 semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços;
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 7 dias realizando trocas do meio a cada 24 horas por meio osteogênico (meio de diferenciação) – DMEM com 10% SFB, suplementado com 50 μg/mL $^{-1}$ de ácido ascórbico e 10 nmol L $^{-1}$ β-glicerofosfato; Sigma/Aldrich Corp;
6. Após o período de 7 dias, aspirar o meio;
7. Pipetar 150μl de detergente Lauryl Sulfato de Sódio a 0,1% (Lise celular);
8. Incubar em temperatura ambiente por 40 minutos;
   OBS: Desses 150μl: 100μl foram usados para o ensaio de PT e 50μl para a Fosfatase Alcalina.
9. São necessários três tubos de ensaio para cada grupo experimental e seis tubos para a curva padrão;
10. Pipetar 50μl do Substrato 1 em todas as amostras (inclusive para todos os tubos da curva padrão);
11. Pipetar 500μl do Substrato 2 em todas as amostras (Observar montagem da curva abaixo);
12. Colocar os tubos em banho-maria por 2 minutos a 37ºC;
13. Pipetar 50μl das amostras em seus referentes tubos de ensaio;
14. Manter os tubos em banho-maria por mais 10 minutos;
15. Pipetar 2 ml do reagente de cor – Reagente 3 do Kit em cada amostra (inclusive na curva padrão);

16. Plaquear em duplicata 100µl das soluções com as amostras em placa de 96 poços;
17. Realizar leitura em 590nm.

OBS: Uma Curva Padrão, com concentrações já conhecidas, é realizada da seguinte forma:

1º Ponto da curva: Branco – 550µl de tampão (Substrato 2) + 50µl (Substrato 1);
2º Ponto da curva: 25µl de fosfatase (Substrato 4) + 525µl de tampão (Substrato 2) + 50µl (Substrato 1) – [1,87 U/L];
3º Ponto da curva: 50µl de fosfatase (Substrato 4) + 500µl de tampão (Substrato 2) + 50µl (Substrato 1) – [3,75 U/L];
4º Ponto da curva: 100µl de fosfatase (Substrato 4) + 450µl de tampão (Substrato 2) + 50µl (Substrato 1) – [7,5 U/L];
5º Ponto da curva: 200µl de fosfatase (Substrato 4) + 350µl de tampão (Substrato 2) + 50µl (Substrato 1) – [15 U/L];
6º Ponto da curva: 400µl de fosfatase (Substrato 4) + 150µl de tampão (Substrato 2) + 50µl (Substrato 1) – [30 U/L];

ANEXO L

ENSAIO DE DEPOSIÇÃO DE NÓDULOS DE MINERALIZAÇÃO - ALIZARIN (7 DIAS)

1. Após realizar o plantio das células semelhantes à odontoblastos MDPC – 23 semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços;
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 7 dias realizando trocas do meio a cada 24 horas por meio osteogênico (meio de diferenciação) – DMEM com 10% SFB, suplementado com 50 μg/mL $^{-1}$ de ácido ascórbico e 10 nmol L $^{-1}$ β-glicerofosfato; Sigma/Aldrich Corp;
6. Após o período de 7 dias, aspirar o meio;
7. Pipetar 100μl de álcool 70% em cada poço (Fixação das Células no fundo do poço);
8. Incubar em temperatura de 4ºC (geladeira) por 1 hora;
9. Aspira o álcool;
10. Lavar uma vez com 100μl água destilada;
11. Aspirar;
12. Pipetar 100μl de Vermelho de Alizarin (40 mmol L $^{-1}$; pH 4,2; ASS 33 PM: 342,26) em cada poço;
13. Agitar por 20 minutos em shaker orbital;
14. Aspirar o meio;
15. Lavar uma vez com 100μl água destilada;
16. Aspirar;
17. Pipetar 200μl de Acetilpiridinium 10% em PBS 1X (Dissolver os Cristais formados pela coloração);
18. Agitar por 15 minutos em shaker orbital;
19. Realizar leitura em 562nm.

ANEXO M

ENSAIO DE EXPRESSÃO DE GENES – PCR EM TEMPO REAL (14 DIAS)

1. Após realizar o plantio das células semelhantes à odontoblastos MDPC – 23 semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços;
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM sem SFB com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 14 dias realizando trocas do meio a cada 24 horas por meio osteogênico (meio de diferenciação) – DMEM com 10% SFB, suplementado com 50 μg/mL $^{-1}$ de ácido ascórbico e 10 nmol L $^{-1}$ β-glicerofosfato; Sigma/Aldrich Corp;
6. Após o período de 14 dias, aspirar o meio;

Extração de RNA

(RNAqueous®-Micro Total RNA Isolation Kit # AM1931 LifeTechnologies)

1. Pipetar 100μl de Tampão de Lise (Lysis Slolution) em cada poço ressuspendendo a amostra;

Obs: pode ser armazenado em temperatura -20ºC, e usar no dia seguinte.

2. Pipetar 100μl da amostra + 50μl de álcool etílico 100% (Sigma Aldrich);
3. Nomear os tubos de filtração (Micro Filter Cartridges e Tubes);
4. Transferir toda a solução para os tubos de filtração;
5.
6. Centrifugar por 10 segundos a 12.000 rpm;
7. Lavar 1X com 180μl da solução 1 do Kit (Wash Solution 1 Concentrate);
8. Centrifugar por 10 segundos a 12.000 rpm;
9. Lavar 2X com 180μl da solução 2/3 do Kit (Wash Solution 2/3 Concentrate);
10. Centrifugar por 10 segundos a 12.000 rpm;
11. Descartar os resíduos das lavagens;
12. Transferir apenas o filtro para o tubo de Eluição/coleta (Micro Elution Tubes);

13. Adicionar 5µl da solução de Eluição (Elution solution) a 75ºC;
14. Centrifugar por 5 minutos a 12.000 rpm;
15. Adicionar mais 5µl da solução de Eluição (Elution solution) a 75ºC;
16. Centrifugar por 5 minutos a 12.000 rpm;
17. Transferir o RNA extraído para mini eppendorfs devidamente identificados;
18. Pipetar 1µl de cada amostra e de H2O ultrapura (Branco) em placas de leitura Take3;
19. Leitura da absorbância (260-Peak, 280-Ration, 320-Ref) Quantificação de Ácido Nucléico.

Síntese de cDNA
(High-Capacity cDNA Reverse Transcription Kits
for 200 Reactions-Applied Biosystems)

1. Inicialmente realizar a preparação de um Mix contendo para cada reação:
   2µl de 10X RT Buffer, 0,8µl de 25X dNTP Mix, 2µl de 10X RT Random Primers, 1µl de MultiScribe Reverse Transcriptase e 4,2µl de H2O ultrapura livre de nuclease.
2. Agitar o Mix;
3. Pipetar 10µl de Mix em cada amostra;
4. Pipetar 0,5µg/µl de cada amostra;
5. Centrifugar por 10 segundos;
6. Inserir os eppendorfs em Termociclador no seguinte ciclo conforme instruções do fabricante:
   25ºC (10min); 37ºC (120min); 85ºC (5min); 4ºC estabilização.

PCR em Tempo Real

1. Preparo do Mix:

   6,25µl de Power Sybr Green PCR Master Mix, 5,75µl de H2O ultrapura livre de nuclease, 1µl de forward (GAPDH, DSSP, DMP-1), e 1µl de reverse (GAPDH, DSSP, DMP-1).

   OBS: Para cada eppendorf colocar 14µl do Mix + 1µl de cada amostra de cDNA.
2. Transferir 10µl para a placa de qPCR;
3. Centrifugar placa de qPCR por 2minutos a 4000rpm;
4. Colocar a placa de qPCR no equipamento StepOnePlus para que se inicie o ciclo.

Curva de qPCR:

Diluição seriada:

1º Ponto da curva: Branco

2º Ponto da curva: 28µl + 2µl da amostra de cDNA

Passar 14µl para o próximo eppendorf contendo 14µl de Mix e assim sucessivamente até completar 6 pontos. Passar 10µl para a placa de qPCR;

ANEXO N

COMITÊ DE ÉTICA (Capítulo 1)

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
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 antimicrobiano de análogos de peptídeos catiônicos sobre microrganismos de importância endodôntica e sua influência na expressão gênica de marcadores de mineralização dentinária"**, Processo FOA nº 2014-00592, 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: 22 de Julho de 2016.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 22 de Agosto de 2016.

CERTIFICATE

We certify that the study entitled **"Antimicrobial effect of cationic peptides analogs on endodontic microrganisms and its influence on gene expression of dentin mineralization markers"**, Protocol FOA nº 2014-00592, 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: July 22, 2016.
DATE OF SUBMISSION OF THE FINAL REPORT: August 22, 2016.

Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

ANEXO O

REFERÊNCIAS INTRODUÇÃO GERAL

1. NCCLS: National Committee for Clinical Laboratory Standards, 2002a. Reference method for broth dilution antifungal susceptibility testing of yeast: approved standard. National Committee for Clinical Laboratory Standards, Wayne, PA, CLSI document M27-A2.
2. NCCLS: National Committee for Clinical Laboratory Standards, 2002b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th ed., Wayne, PA, National Committee for Clinical Laboratory Standards, CLSI document M7-A5.
3. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Micros 2003; Dec 36(4);247-252.
4. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dent Traumatol. 2002 Jun;18(3):134-7.
5. Baik JE, Ryu YH, Han JY, Im J, Kum KY, Yun CH, Lee K, Han SH. Lipoteichoic acid partially contributes to the inflammatory responses to Enterococcus faecalis. J Endod. 2008 Aug;34(8):975-82.
6. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod. 2004 Apr;30(4):196-200.
7. Byström A, Sundqvist G (1981). Bacteriological evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 89:321-328.
8. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J. 1985 Jan;18(1):35-40.
9. Chávez De Paz LE, Dahlén G, Molander A, Möller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J. 2003 Jul;36(7):500-8.
10. Ciardi JE, Rölla G, Bowen WH, Reilly JA. Adsorption of *Streptococcus mutans* lipoteichoic acid to hydroxyapatite. Scand J Dent Res. 1977 Sep;85(6):387-91.
11. da Silva LA, Nelson-Filho P, da Silva RA, Flores DS, Heilborn C, Johnson JD, Cohenca N. Revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing in dogs' teeth with apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010 May;109(5):779-87.
12. de Oliveira LD, Jorge AO, Carvalho CA, Koga-Ito CY, Valera MC. In vitro effects of endodontic irrigants on endotoxins in root canals. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007 Jul;104(1):135-42.

13. Deng DM, Buijs MJ, ten Cate JM.The effects of substratum on the pH response of *Streptococcus mutans* biofilms and on the susceptibility to 0.2% chlorhexidine. Eur J Oral Sci. 2004 Feb;112(1):42-7.
14. Ding RY, Cheung GS, Chen J, Yin XZ, Wang QQ, Zhang CF. Pulp revascularization of immature teeth with apical periodontitis: a clinical study. J Endod. 2009 May;35(5):745-9.
15. Doyon GE, Dumsha T, von Fraunhofer JA. Fracture resistance of human root dentin exposed to intracanal calcium hydroxide. J Endod. 2005 Dec;31(12):895-7.
16. Gorr SU, Aldolhosseini M. Antimicrobial peptides and periodontal disease. J Clin Periodontal. 2011; 38 Suppl 11:126-41.
17. Han SH, Kim JH, Martin M, Michalek SM, Nahm MH. Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. Infect Immun. 2003 Oct;71(10):5541-8.
18. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem. 2001 Feb 23;276(8):5707-13.
19. Hartke A, Giard JC, Laplace JM, Auffray Y. Survival of *Enterococcus faecalis* in an oligotrophic microcosm: changes in morphology, development of general stress resistance, and analysis of protein synthesis. Appl Environ Microbiol. 1998 Nov;64(11):4238-45.
20. Hölzl MA, Hofer J, Steinberge P, Pfistershammer K, Zlabinger GJ. Host anticrobial proteins as endogenous immunomodulators. Immunol Lett. 2008;119(1-2):4-11.
21. Iglesias-Linares A, Yáñez-Vico RM, Sánchez-Borrego E, Moreno-Fernández AM, Solano-Reina E, Mendoza-Mendoza A. Stem cells in current paediatric dentistry practice. Arch Oral Biol. 2013 Mar;58(3):227-38.
22. Ishizaka R, Iohara K, Murakami M, Fukuta O, Nakashima M. Regeneration of dental pulp following pulpectomy by fractionated stem/progenitor cells from bone marrow and adipose tissue. Biomaterials. 2012 Mar;33(7):2109-18.
23. Jacob B, Park IS, Bang JK, Shin SY.Short KR-12 analogs designed from human cathelicidin LL37 possessing both antimicrobial and antiendotoxicactivities without mammalian cell toxicity. J Pept Sci. 2013 Nov;19(11):700-7.
24. Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y. Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. J Periodontal Res. 2007 Oct;42(5):410-9.
25. Jönsson D, Nilsson BO. The antimicrobial peptide LL-37 is anti-inflammatory and proapoptotic in human periodontal ligament cells. J Periodontal Res. 2012 Jun;47(3):330-5.
26. Jung IY, Lee SJ, Hargreaves KM. Biologically based treatment of immature permanent teeth with pulpal necrosis: a case series. J Endod. 2008 Jul;34(7):876-87.
27. Kajiya M, Shiba H, Komatsuzawa H, Ouhara K, Fujita T, Takeda K, Uchida Y, Mizuno N, Kawaguchi H, Kurihara H. The antimicrobial peptide LL37 induces the

migration of human pulp cells: a possible adjunct for regenerative endodontics. J Endod. 2010 Jun;36(6):1009-13.

28. Karanxha L, Park SJ, Son WJ, Nör JE, Min KS.Combined effects of simvastatin and enamel matrix derivative on odontoblastic differentiation of human dental pulp cells. J Endod. 2013 Jan;39(1):76-82.
29. Kittaka M, Shiba H, Kajiya M, Fujita T, Iwata T, Rathvisal K, Ouhara K, Takeda K, Fujita T, Komatsuzawa H, Kurihara H. The antimicrobial peptide LL37 promotes bone regeneration in a rat calvarial bone defect. Peptides. 2013 Aug;46:136-42.
30. Kraus D, Deschner J, Jäger A, Wenghoefer M, Bayer S, Jepsen S, Allam JP, Novak N, Meyer R, Winter J. Human β-defensins differently affect proliferation, differentiation, and mineralization of osteoblast-like MG63 cells. J Cell Physiol. 2012 Mar;227(3):994-1003.
31. Lee JK, Chang SW, Perinpanayagam H, Lim SM, Park YJ, Han SH, Baek SH, Zhu Q, Bae KS, Kum KY. Antibacterial Efficacy of a Human β-Defensin-3 Peptide on Multispecies Biofilms. J Endod. (2013b) Dec;39(12):1625-9.
32. Lee JK, Park YJ, Kum KY, Han SH, Chang SW, Kaufman B, Jiang J, Zhu Q, Safavi K, Spångberg L. Antimicrobial efficacy of a human β-defensin-3 peptide using an Enterococcus faecalis dentine infection model. Int Endod J. (2013a). May;46(5):406-
33. Lee SH, Baek DH.Antibacterial and neutralizing effect of human β-defensins on Enterococcus faecalis and Enterococcus faecalis lipoteichoic acid. J Endod. 2012 Mar;38(3):351-6.
34. Lee SH, Jun HK, Lee HR, Chung CP, Choi BK. Antibacterial and lipopolysaccharide (LPS)-neutralising activity of human cationic antimicrobial peptides against periodontopathogens. Int J Antimicrob Agents. 2010 Feb;35(2):138-45.
35. Li W, Liu H, Xu Q. Extracellular dextran and DNA affect the formation of Enterococcus faecalis biofilms and their susceptibility to 2%chlorhexidine. J Endod. 2012 Jul;38(7):894-8.
36. Mccormick TS, Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity Periodontology 2000, 2010;Oct 54(1):195–206.
37. Mor A, Hani K, Nicolas PJ. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 1994;269:31635–41.
38. Moreno-Hidalgo MC, Caleza-Jimenez C, Mendoza-Mendoza A, Iglesias-Linares A. Revascularization of immature permanent teeth with apical periodontitis. Int Endod J. 2013 Jul 3.
39. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004 Nov 1;15(6):348-81.
40. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M. Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, {beta}-defensins and LL37,

produced by human epithelial cells. J Antimicrob Chemother. 2005 Jun;55(6):888-96.

41. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review-Part I: chemical, physical, and antibacterialproperties. J Endod. 2010 Jan;36(1):16-27.
42. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Ver Biochem. 2002;71:635-700. Epub 2001 Nov 9. Review.
43. Rafter M. Apexification: a review. Dent Traumatol. 2005 Feb;21(1):1-8.
44. Sakamoto M, Siqueira JF Jr, Rôças IN, Benno Y. Bacterial reduction and persistence after endodontic treatment procedures. Oral Microbiol Immunol. 2007 Feb;22(1):19-23.
45. Shabahang S, Torabinejad M. Treatment of teeth with open apices using mineral trioxide aggregate. Pract Periodontics Aesthet Dent. 2000 Apr;12(3):315-20; quiz 322.
46. Sheehy EC, Roberts GJ.Use of calcium hydroxide for apical barrier formation and healing in non-vital immature permanent teeth: a review. Br Dent J. 1997 Oct 11;183(7):241-6.
47. Shiba H, Mouri Y, Komatsuzawa H, Ouhara K, Takeda K, Sugai M, Kinane DF, Kurihara H. Macrophage inflammatory protein-3alpha and beta-defensin-2 stimulate dentin sialophosphoprotein gene expression in human pulp cells. Biochem Biophys Res Commun. 2003 Jul 11;306(4):867-71.
48. Siqueira JF Jr, Magalhães KM, Rôças IN. Bacterial reduction in infected root canals treated with 2.5% NaOCl as an irrigant and calcium hydroxide/camphorated paramonochlorophenol paste as an intracanal dressing. J Endod. 2007 Jun;33(6):667-72.
49. Siqueira JF Jr, Paiva SS, Rôças IN. Reduction in the cultivable bacterial populations in infected root canals by a chlorhexidine-based antimicrobialprotocol. J Endod. 2007 May;33(5):541-7. Epub 2007 Mar 12.
50. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009 Nov;88(11):969-81. doi: 10.1177/0022034509346549. Appendix
51. Siqueira JF Jr, Rôças IN, Rosado AS. Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. Oral Microbiol Immunol. 2004 Dec;19(6):363-70.
52. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 2--Redefining the endodontic microbiota. J Endod. 2005 Jul;31(7):488-98. Review.
53. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J. 1999 Sep;32(5):361-9. Review.
54. Sjögren U, Figdor D, Persson S, Sundqvist G.Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J. 1997 Sep;30(5):297-306.

55. Song W, Shi Y, Xiao M, Lu H, Qu T, Li P, Wu G, Tian Y.In vitro bactericidal activity of recombinant human beta-defensin-3 against pathogenic bacterial strains in humantooth root canal. Int J Antimicrob Agents. 2009 Mar;33(3):237-43.
56. Suphasiriroj W, Mikami M, Shimomura H, Sato S. Specificity of antimicrobial peptide LL-37 to neutralize periodontopathogenic lipopolysaccharide activity in human oral fibroblasts. J Periodontol. 2013 Feb;84(2):256-64. doi: 10.1902/jop.2012.110652.
57. Taneja S, Kumari M, Parkash H. Nonsurgical healing of large periradicular lesions using a triple antibiotic paste: A case series. Contemp Clin Dent. 2010 Jan;1(1):31-5.
58. Taylor K, Clarke DJ, McCullough B, Chin W, Seo E, Yang D, Oppenheim J, Uhrin D, Govan JR, Campopiano DJ, MacMillan D, Barran P, Dorin JR. Analysis and separation of residues important for chemoattractant and antimicrobial activities of beta-defensin3. J Biol Chem. 2008 Mar 14;283(11):6631-9.
59. Thibodeau B, Teixeira F, Yamauchi M, Caplan DJ, Trope M. Pulp revascularization of immature dog teeth with apical periodontitis. J Endod. 2007 Jun;33(6):680-9. Epub 2007 Apr 24.
60. Vianna ME, Conrads G, Gomes BP, Horz HP. Identification and quantification of archaea involved in primary endodontic infections. J Clin Microbiol. 2006 Apr;44(4):1274-82.
61. Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J, et al. Osteoimmunology: interplay between the immune system and bone metabolism. Annu Rev Immunol. 2006;24:33-63.
62. Wang G.Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J Biol Chem. 2008 Nov 21;283(47):32637-43.
63. Wang X , Thibodeau B , M Trope , Lin LM , Huang GT . Histologic characterization of regenerated tissues in canal space after the evitalization/revascularizationprocedure of immature dog teeth with apical periodontitis. J Endod 2010 Jan; 36 (1) :56-63.
64. Wiesner J, Vilcinskas A. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 2010 Sep/Oct;1(5):440-464.
65. Wong JH, Ng TB, Legowska A, Rolka K, Hui M, Cho CH. Antifungal action of human cathelicidin fragment (LL13-37) on Candida albicans. Peptides. 2011 Oct;32(10):1996-2002. doi: 10.1016/j.peptides.2011.08.018. Epub 2011 Aug 26.
66. Zhang Z, Cherryholmes G, Shively JE. Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin.J Leukoc Biol. 2008;Oct 84(3):780-8.
67. Zhang Z, Shively JE. Generation of novel bone forming cells (monoosteophils) from the cathelicidin-derived peptide LL-37 treated monocytes. PLoS One. 2010 Nov 15;5(11):e13985. doi: 10.1371/journal.pone.0013985.