UNESP – Universidade Estadual Paulista

Faculdade de Odontologia de Araraquara

TELMA BLANCA LOMBARDO BEDRAN

"Efeito antimicrobiano e modulador da resposta imune dos peptídeos hBD-3 e LL-37 e dos polifenóis do chá verde e do cranberry"

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Orientadora: Profa. Dra. Denise M. Palomari Spolidorio

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TELMA BLANCA LOMBARDO BEDRAN

"EFEITO ANTIMICROBIANO E MODULADOR DA RESPOSTA IMUNE DOS PEPTÍDEOS hBD-3 E LL-37 E DOS POLIFENÓIS DO CHÁ VERDE E DO CRANBERRY"

COMISSÃO JULGADORA
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DEDICATÓRIA

Dedico mais esta vitória...

Á Deus

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Resumo

Bedran TBL. Efeito antimicrobiano e modulador da resposta imune dos peptídeos hBD-3 e LL-37 e dos polifenóis do chá-verde e do cranberry [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2010.

Resumo

Os peptídeos antimicrobianos como por exemplo a catelicina (LL-37) e as defensinas humanas (hBD-1, hBD-2 e a hBD-3) são considerados antibióticos endógenos com importante papel na prevenção das doenças periodontais, devido a sua capacidade de regulação da resposta imune, sendo que os mesmos podem ser degradados pelos periodontopatógenos. Terapias que aumentem a produção destes peptídeos pelas próprias células do organismo, assim como a associação destes peptídeos com compostos naturais os quais possam agir em sinergismo na regulação da resposta imune, podem ser considerados novas estratégias para o melhor controle das doenças periodontais. Portanto os objetivos deste estudo in vitro foram: i) Avaliar a capacidade do extrato do chá verde (*Camellia sinensis*) e do seu polifenol, o EGCG, sobre a expressão gênica de hBD-1 e hBD-2 pelas células epiteliais gengivais (B11), sobre a degradação das mesmas frente ao *P. gingivalis*, ii) Através da utilização do modelo 3D de co-cultura celular, avaliar a capacidade anti-inflamatória dos peptídeos hBD-3 e LL-37 quando em associação sobre a produção de citocinas, quimiocinas e fatores de crescimento, iii) Avaliar a capacidade anti-inflamatória da associação do EGCG e do polifenol proveniente do cranberry, o AC-PACs, com o peptídeo LL-37 sobre a produção de citocinas, quimiocinas e fatores de crescimento em modelo de co-cultura celular. As células epiteliais gengivais (B11) foram estimuladas com o extrato do chá verde e com o EGCG na presença e ausência de inibidores específicos. A produção e expressão gênica de hBD-1 e hBD-2 foram quantificados respectivamente pelas técnica de ELISA e qPCR. A capacidade do extrato do chá verde e do EGCG em proteger a degradação de hBDs pelo *P. gingivalis* foi mensurado através da técnica de ELISA. Foi desenvolvido um modelo em 3D de co-cultura de fibroblastos gengivais embebidos em uma matriz de colágeno com células epiteliais gengivais

semeadas em sua superfície, no qual observou-se efeito sinérgico das células na secreção de IL-6 e IL-8 em resposta ao estímulo com LPS de *A. actinomycetemcomitans* (1 μg/ml) quando comparados as células individuais. Em seguida o modelo em 3D de co-cultura celular na presença no LPS de *A. actinomycetemcomitans* foi estimulado com: i) hBD-3 (10 and 20 μM) e LL-37 (0.1 and 0.2 μM), ii) EGCG (1 e 5 μg/ml) e LL-37 (0.1 and 0.2 μM) e iii) AC-PACs (25 e 50 μg/ml) e LL-37 (0.1 and 0.2 μM), individualmente e em associação. Foi utilizada a técnica de ELISA Multiplex para quantificar 41 diferentes citocinas, quimiocinas, fatores de crescimentos e 13 diferentes MMPs e TIMPs. Os resultados demonstraram que: i) o extrato do chá verde e o EGCG aumentaram a produção e a expressão gênica de hBD-1 e hBD-2 pelas células epiteliais de uma maneira dose-dependente, e foram capazes de prevenir a degradação das hBD-1 e hBD-2 recombinantes pelo sobrenadante de *P. gingivalis*, ii) o peptídeo hBD-3 em associação com o LL-37 mostrou efeito sinérgico na diminuição da produção de GRO-α, G-CSF, IP-10, IL-6, e MCP-1, entretanto teve apenas efeito aditivo na redução da produção de IL-8 em resposta ao estímulo com LPS de *A. actinomycetemcomitans,* iii) a associação do peptídeo LL-37 com o EGCG e com o AC-PACs mostraram efeito sinérgico na redução da produção de GRO-α, G-CSF e IL-6 em resposta ao estímulo com LPS de *A. actinomycetemcomitans.* Portanto os peptídeos antimicrobianos hBD-3 e LL-37, assim como o extrato do chá verde, o EGCG e o AC-PACs por demonstrarem capacidade de regular a produção de citocinas inflamatórias, induzir a produção de defensinas pelas próprias células e proteger as defensinas da degradação pelo *P. gingivalis* surgem como promissores alternativas para terapias adjuntas ao tratamento periodontal convencional. Entretanto estudos clínicos futuros são necessários para avaliar o papel dos peptídeos e dos compostos naturais na cavidade oral e na proteção dos tecidos periodontais frente à uma agressão.

Palavras-chave: bactérias, *Camellia sinensis*, cranberry, Ensaio de imunoadsorção enzimática, peptídeos.

Abstract

Bedran TBL. Antimicrobial and immune regulatory activity of the peptides hBD-3, LL-37 and the polyphenols from cranberry and green tea [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2010.

Abstract

The antimicrobial peptides LL-37, hBD-1, hBD-2 and hBD-3 are considered an endogenous antibiotic, with important role in the prevention of periodontal diseases due to their ability to regulate the immune response. However those peptides could be degraded by periodontal pathogens. Therefore, therapies able to up regulate the secretion of those peptides by human cells, and the association of antimicrobial peptides with natural compounds, which may act in synergism to modulate the immune response, may be a novel approach for effectively controlling periodontal diseases. The aim of this in vitro study were: i) investigate the ability of green tea extract and EGCG to induce hBD-1 and hBD-2 secretion and gene expression by gingival epithelial cells (B11) and to protect hBDs from degradation by *P. gingivalis*, ii) A 3D co-culture model of gingival epithelial cells and fibroblasts stimulated with *A. actinomycetemcomitans* LPS (1 μg/ml) were used to investigated the anti-inflammatory properties of the hBD-3, LL-37, AC-PACs and EGCG and to determine whether LL-37 acts in synergy with AC-PACs, EGCG and hBD-3. Gingival epithelial cells were stimulated with green tea extract or EGCG in the presence and absence of specific inhibitors. The secretion and gene expression of hBD-1 and hBD-2 was respectively measured by ELISA and qPCR. The ability of green tea extract and EGCG to prevent hBDs degradation by *P. gingivalis* present in a bacterial culture supernatant was evaluated by ELISA. A 3D co-culture model composed of gingival fibroblasts embedded in a collagen matrix overlaid with gingival epithelial cells had a synergistic effect with respect to the secretion of IL-6 and IL-8 in response to *A. actinomycetemcomitans* LPS stimulation compared to fibroblasts and epithelial cells individually. The 3D co-culture model was stimulated with non-cytotoxic concentrations of: i) hBD-3 (10 and 20 μM) and LL -37 (0.1 and 0.2 μM), ii) EGCG (

1 and 5 μg/ml) and LL -37 (0.1 and 0.2 μM), iii) AC- PACs (25 and 50 μg/ml) and LL-37 (0.1 and 0.2 μM) alone and in association in the presence of *A. actinomycetemcomitans* LPS. A multiplex ELISA assay was used to quantify the secretion of 41 different cytokines, chemokines, growth factors and 13 different MMPs and TIMPs. Ours results showed that: i) the secretion and gene expression of hBD-1 and hBD-2 was up-regulated in a dose-dependent manner by green tea extract and EGCG. Green tea extract and EGCG were also able to prevent the degradation of recombinant hBD-1 and hBD-2 by a culture supernatant of *P. gingivalis*, ii) hBD-3 in association with LL-37 showed a synergistic effect to reduce the secretion of GRO- α, G-CSF, IP- 10, IL -6 and MCP -1, however had only additive effect on reducing the secretion of IL-8 in response to *A. actinomycetemcomitans* LPS stimulation, iii) the association of the peptide LL- 37 with EGCG and with AC- PACs showed a synergistic effect to reduce the secretion of GRO-α, G-CSF and IL-6 in response to *A. actinomycetemcomitans* LPS stimulation. Considering that the antimicrobial peptides hBD-3 and LL-37, as well as green tea extract, EGCG and AC- PACs, demonstrated the ability to regulate the secretion of inflammatory cytokines, up regulated the secretion of defensins by the cells and even to protect the defensins degradation by *P. gingivalis*, emerged as promising alternative adjunct therapy to conventional periodontal treatment. However future clinical studies are necessary to evaluate the role of peptides and natural compounds in the oral cavity and periodontal tissues.

Keywords: Peptides, bacteria, ELISA, *Camellia sinensis*, cranberry.

Lista de abreviaturas

LISTA DE ABREVIATURAS E SIGLAS

3D: three-dimensional/três-dimensões

AC-PACs: A-type cranberry proanthocyanidins/proantocianidina do tipo A

Aggregatibacter actinomycetemcomitans: *A. actinomycetemcomitans*

AMPs: antimicrobial peptides

EGCG: epigallocatechin-3gallate/epigalocatequina galato

G-CSF: granulocyte colony-stimulating factor

Gram-negativas/Gram positivas: G-/G+

GRO-α: CXC-chemokine ligand 1

hBDs: human beta-defensin/beta-defensina humana

IL: interleukin/interleucina

IP-10: interferon-γ inducible protein 10

LL-37: cathelicidin/catelicina

LPS: lipopolysaccharide/lipopolissacarídeo

MCP-1: monocyte chemoattractant protein 1

MMPs: matrix metalloproteinase/ metaloproteinases de matriz

PAMs: peptídeos antimicrobianos

Porphyromonas gingivalis: *P. gingivalis*

qPCR: polymerase chain reaction/reação em cadeia da polimerase

TIMPs: tissue inhibitors of metalloproteinases

TLR: toll like receptors/receptores do tipo Toll

TNF-α: Tumor necrosis factor α/Fator de necrose tumoral α

Sumário

SUMÁRIO

Introdução

INTRODUÇÃO E RELEVÂNCIA CLÍNICA

A periodontite é uma inflamação crônica iniciada pela presença de uma infecção microbiana variada a qual leva a destruição dos tecidos de suporte do dente, tais como gengiva, osso, cemento e ligamento periodontal[52]. A periodontite possui como principais fatores etiológicos, a presença de bactérias G- e a ativação da resposta imune do hospedeiro em resposta a invasão microbiana[68]. Dentre as bactérias G-, o *P. gingivalis* é considerado um dos principais fatores etiológicos da periodontite crônica e o *A. actinomycetemcomitans* da periodontite agressiva. Essas bactérias possuem como fator de virulência a presença do LPS [23, 50, 56]. O LPS está presente na membrana externa das bactérias G- e possui a capacidade de aderir a receptores presentes nas células do hospedeiro e com isso gerar uma resposta imune inflamatória exacerbada e consequentemente à destruição dos tecidos periodontais[4,5]. Com isso o LPS é considerado um importante fator de virulência bacteriano e causador de doenças infecciosas e, são considerados potentes alvos terapêuticos[38].

A redução dos patógenos e seus fatores de virulência e consequentemente a modulação da resposta imune do hospedeiro, seja através da diminuição da produção de citocinas inflamatórias ou do aumento da imunidade do hospedeiro frente à uma agressão tornam-se pré-requisitos para a resolução das doenças periodontais e a manutenção da saúde oral[49, 52, 53, 70, 76].

Nos tecidos periodontais, os dois tipos celulares predominantes são as células epiteliais e os fibroblastos, os quais formam uma barreira mecânica contra a invasão dos periodontopatógenos e seus fatores de virulência[76]. Estes tipos celulares são a primeira defesa do organismo durante os processos infecciosos e considerados de suma importância nos processos inflamatórios orais, como por exemplo, na periodontite[76]. O objetivo das células é impedir a progressão da doença e a tentativa de eliminação dos periodontopatógenos e remodelação dos tecidos orais através da produção de citocinas e peptídeos antimicrobianos[76]. Estas células frente à uma agressão secretam elevado número de citocinas inflamatórias, quimiocinas, fatores de crescimento e MMPs com o intuito de conter a inflamação e eliminar ou inativar os patógenos

presentes. Assim, a resposta imune exacerbada frente à uma agressão, irá elevar as citocinas inflamatórias as quais irão levar a destruição dos tecidos periodontais[25, 46, 63, 71].

Com isso a primeira estratégia para manter a boa saúde oral e assim prevenir a formação do biofilme bacteriano é inibir o potencial patogênico dos periodontopatógenos e seus fatores de virulência, assim como lançar mão de terapias que regulem a resposta imune/inflamatória do hospedeiro visando modular o binômio formação/destruição dos tecidos de sustentação do órgão dentário[28, 31].

A terapia de redução bacteriana por meio do controle mecânico do biofilme dentário, assim como da terapia básica de raspagem e alisamento radicular manual, são tratamentos que apresentam bons índices de sucesso e ainda é a terapia mais indicada na prática clínica. Contudo existem casos em que esta abordagem não é suficiente, exigindo complementações à terapia tradicional, podendo-se utilizar terapias coadjuvantes focada na tentativa de modular a resposta do hospedeiro e assim tentar suprimir a destruição dos tecidos periodontais pela reação inflamatória crônica[28, 31].

Devido o crescente número de resistência bacteriana aos antibióticos[40, 72], novas estratégias terapêuticas têm sido foco de estudo, no intuito também de modular a resposta do hospedeiro e interromper a destruição dos tecidos periodontais[28].

Considerando a característica multifatorial das doenças periodontais, a importância dos PAMs para a proteção dos tecidos periodontais e a resistência bacteriana aos antibióticos, a associação de diferentes drogas são pontos que surgem como uma nova estratégia de tratamento alternativa para solucionar estes problemas e consequentemente obter melhor resposta dos tecidos aos tratamentos periodontais[28, 72]. A associação de diferentes drogas pode ser útil para eliminar múltiplos patógenos ao mesmo tempo, ou até mesmo eliminar um só tipo de patógeno porém com uma concentração menor e melhores respostas do tratamento. Assim, as principais características da associação de drogas que possam agir em sinergismo é i) utilizar menores concentrações das

drogas com o mesmo ou melhor resposta dos tecidos ao tratamento ii) diminuir a resistência às drogas gerada pelos microrganismos[15].

Os PAMs são pequenas moléculas de natureza catiônica com capacidade hidrofóbica, considerados antibióticos produzidos endogicamente, os quais representam importantes componentes da resposta imune inata dos organismos celulares[9, 17, 48, 55]. Têm sido demonstrado que os PAMs são contribuintes importantes para manutenção do equilíbrio entre saúde e doença no complexo meio da cavidade oral[12, 72].

Apesar do grande número de PAMs existentes na natureza (de acordo com o banco de dados de peptídeos de antimicrobianos http://aps.unmc.edu/AP há aproximadamente 1700 tipos de peptídeos), as duas principais classes de peptídeos presentes nos humanos são as defensinas e as catelicinas[12]. Estas duas classes de peptídeos possuem além do efeito antimicrobiano, potente efeito anti-inflamatório de inativação do LPS bacteriano[26, 29, 55].

Na família das catelicinas apenas uma cópia do gene está presente nos humanos, também conhecida como CAMP- ou CAP18[12]. A extremidade C-terminal desse peptídeo sofre proteólise gerando um pequeno peptídeo de 37 aminoácidos, o qual se inicia com dois resíduos de leucina, sendo a partir disso chamado de LL-37. O LL-37 é um peptídeo catiônico, com amplo espectro de ação, predominantemente encontrado e secretado em vários tecidos e fluidos, tais como vias aéreas, neutrófilos polimorfonucleares, células epiteliais e saliva[12, 19, 20, 39]. Nas bactérias G-, o LL-37 possui a capacidade de penetrar no espaço periplasmático e interromper o crescimento bacteriano[12, 27, 49, 65, 67].

O peptídeo LL-37 também possui a capacidade de neutralizar o LPS bacteriano, através do bloqueio da ligação do LPS à seu receptor de membrana celular, inibindo assim a via de sinalização celular desencadeada por ligantes do tipo TLR[12, 39] .

Avaliando o efeito de antibióticos assim como do LL-37 na inibição da produção de citocinas pró-inflamatórias (TNF-α, IL-6 e IL-1β) induzido por LPS de *P. gingivalis* em células do sangue total, Walters et al.[72] (2010) encontraram que o peptídeo LL-37 pode ser efetivo na

redução de citocinas estimuladas por LPS, concluindo que o LL-37 pode ser considerado uma terapia de escolha para o tratamento coadjuvante da periodontite. Entretanto foi demonstrado que a concentração de LL-37 encontrado na saliva e no fluido crevicular poderia não ser suficiente para eliminar as bactérias e, concluindo portanto que a concentração necessária para ser efetivo deveria ser maior[3, 30, 69]. Outros autores descreveram que menores concentrações do LL-37 foram encontrados em sítios com doença periodontal, quando comparados à sítios sadios[11, 33, 41].

As defensinas são um grupo de cisteínas, e nos humanos o tipo predominante são as beta-defensinas, as quais são codificadas por vários genes e podem ser expressas por inúmeras células dentre elas as epiteliais[12].

As principais beta-defensinas encontradas nos humanos são as hBD-1, hBD-2 e hBD-3, as quais são expressas na forma constitutiva ou induzida em resposta à um estímulo bacteriano. As hBDs possuem a capacidade de interagir com a membrana bacteriana, levando a formação de poros e consequentemente a lise bacteriana[16, 32, 42, 44, 45]. Merece destaque a hBD-3, que além de ser expressa por células epiteliais também pode ser expressa em baixas concentrações em outros tecidos, como na saliva e fluido crevicular, e possui amplo espectro de ação levando à morte ou eliminação de fungos e bactérias[12, 26, 29, 55, 73].

Além de atuarem como agente antimicrobiano, elas também possuem atividade imunomodulatória de quimiotaxia e a capacidade de atenuar a resposta pró-inflamatória, por meio de diversos mecanismos, dentre eles a ligação do peptídeo à adesina presente na superfície bacteriana[12, 55, 62].

Funderburg et al.[24] (2007) demonstraram que o peptídeo hBD-3 é capaz de inibir a ligação do LPS ao receptor de superfície do tipo TLR e assim modular a resposta do hospedeiro, enquanto outros trabalhos demonstraram redução de hBD-3 no fluido crevicular de pacientes com periodontite crônica. Esta redução pode estar associada ao fato que alguns periodontopatógenos, como o *P. gingivalis,* possuem a capacidade de degradar as defensinas humana[41, 66].

A hBD-3 e o LL-37 podem atuar em associação com outros agentes antimicrobianos para aumentar a atividades antibacterianas e diminuir a degradação pelas bactérias orais[54, 57 58]. Chen et al.[13] (2005) demonstraram que a defensina hBD-3 pode agir em sinergismo com o LL-37 contra *Staphylococcus aureus.* Este fato pode ser explicado devido a capacidade desses peptídeos atuarem por via de sinalização diferentes e consequentemente a hBD-3 e o LL-37 podem ter efeito sinérgico em associação para aumentar a capacidade antimicrobiana.

Levando-se em consideração os efeitos benéficos dos peptídeos hBD-3 e LL-37, como por exemplo a capacidade antimicrobiana e anti-inflamatória benéfica destes peptídeos para a saúde, a capacidade dos mesmos de atuarem por diferentes vias de sinalização intra-celulares e consequentemente inibirem a ação de patógenos, assim como o fato de concentrações reduzidas destes peptídeos estarem associados com a severidade da doença periodontal, surgem como alternativas para melhorar as condições periodontais a: i) utilização de condições que levem ao aumento dos peptídeos pelas próprias células e consequentemente terão impacto positivo na saúde periodontal ii) associação destes peptídeos assim como da associação com compostos naturais que possuam consequentemente efeito sinérgico na redução de citocinas inflamatórias.

O cranberry (*Vaccinium macrocarpon* Ait.) é uma fruta proveniente da América do Norte com efeitos benéficos na saúde humana, principalmente no que diz respeito às doenças infecciosas. Os polifenóis provenientes do cranberry merecem atenção especial, por serem considerados potentes agentes terapêuticos para prevenir diversas doenças, como por exemplo, o câncer, doenças cardiovasculares e doenças infecciosas, como a periodontite[2, 37, 60]. Os principais polifenóis do cranberry são as proantocianidinas do tipo A (AC-PACs). A literatura demonstrou que o AC-PACs são os responsáveis pela atividade biológica do cranberry, por possuir a capacidade de inibir a formação de biofilme bacterianos, enzimas destrutivas dos tecidos periodontais e, até mesmo a diminuição da secreção de citocinas inflamatórias[6-8, 21]. Feldman, Grenier[22] (2012) relataram efeito sinérgico da associação de AC-PACs com outro composto chamado Licochalcone A para reduzir a secreção de IL-1β, TNF-α, IL-6 e IL-8 por macrófagos

estimulados com LPS de *A. actinomycetemcomitans*. Assim, concluíram que a associação deste compostos em baixas concentrações possuem efeito sinérgico para modular a resposta imune do hospedeiro e, consequentemente poderia ser uma abordagem terapêutica interessante para o tratamento das doenças periodontais.

O chá é uma infusão aromática aquosa de folhas secas da planta *Camellia sinensis*. É a segunda bebida mais consumida no mundo após a água. O chá contém inúmeros compostos, como por exemplo, os aminoácidos, os polissacarídeos, vitaminas e polifenóis, sendo os flavonoides a sua maioria[14]. O chá verde é composto por folhas não fermentadas com alta concentração de catequina, incluindo o EGCG[14]. Há evidências que os polifenóis provenientes do chá verde podem contribuir para a redução do risco de doença cardiovascular, câncer e uma variedade de outros efeitos benéficos para a saúde[10, 18]. Entretanto há poucas evidências do efeito benéfico do chá na cavidade oral[34, 35, 44, 47]. Estudos de Koyama et al.[44] (2010) realizado no Japão, mostraram diminuição da perda dentária em indivíduos que consumiram o chá verde, outro estudo de Hamilton-Miller[34] (2001) demonstraram que o consumo de chá verde reduz a incidência de cárie. Além disso, um estudo epidemiológico mostrou que o consumo de uma ou mais xícaras de chá verde durante o dia é capaz de reduzir o risco das doenças periodontais[47].

Estudos clínicos mostraram que a liberação local de catequinas do chá verde em bolsas periodontais aumentam a eficácia dos tratamentos periodontais convencionais[35]. O EGCG é a principal catequina presente no chá verde com diferentes efeitos benéficos na saúde e é capaz de reduzir a secreção de citocinas pró-inflamatórias, interferir na formação de osteoclastos e inibir a produção de proteínas presentes nos processos inflamatórios[1, 36, 51, 61, 74, 75]. Nakanishi et al.[59] (2010) relataram que o EGCG foi capaz de reduzir significativamente a secreção de IL-6 e IL-8 em resposta aos estímulos com LPS de *Escherichia coli* e *Staphylococcus aureus* peptidoglicano. Hirasawa et al.[35] (2002) relataram que a liberação local de EGCG no interior das bolsas periodontais poderia aumentar o sucesso do tratamento periodontal convencional.

Apesar de diferentes estudos comprovarem o efeito benéfico do chá verde, do cranberry e de seus compostos para a saúde periodontal, ainda necessita-se estabelecer como que eles exercem esses efeitos benéficos. Neste sentido a utilização de compostos naturais possuem vantagens em relação aos antibióticos por não produzirem resistência bacteriana. A associação de compostos naturais com peptídeos antimicrobianos, assim como a associação de peptídeos antimicrobianos que possuam diferentes mecanismos de ação surgem como uma nova estratégia para tentar modular a resposta do hospedeiro.

Considerando a importância dos peptídeos antimicrobianos no processo saúde-doença periodontal e levando-se em consideração que eles podem ser degradados pelas bactérias, outra alternativa, é a utilização de estratégias que levem ao aumento da produção de peptídeos do hospedeiro. Portanto peptídeos antimicrobianos e compostos naturais com efeitos benéficos para a saúde oral emergem como candidatos no tratamento coadjuvante, considerando a capacidade dos peptídeos de agirem não somente contra bactérias mas também devido a sua habilidade de regular a resposta imune-inflamatória[64, 72].

Assim, permanece esta lacuna para saber se a associação do peptídeo hBD-3, do EGCG e do AC-PACs com o peptídeo LL-37 potencializa o efeito anti-inflamatório contra o LPS bacteriano, assim como, se o extrato do chá verde possui a capacidade de aumentar a produção de hBD-1 e hBD-2 pelas próprias células do hospedeiro e impedir a degradação das mesmas pelo *P. gingivalis*.

Proposição

PROPOSIÇÃO

Com base nos dados apresentados neste trabalho, temos as seguintes hipóteses:

1- O extrato do chá verde e seu principal polifenol, o EGCG, possuem a capacidade de aumentar a produção de defensinas pelos tecidos epiteliais orais e impedir a degradação das mesmas pelos periodontopatógenos;

2- As células epiteliais e os fibroblastos agem em sinergismo frente à uma agressão com LPS bacteriano;

3- Os peptídeos antimicrobianos hBD-3 e LL-37 possuem capacidade anti-inflamatória e efeito sinérgico quando em associação, na regulação de citocinas inflamatórias, frente à uma agressão com LPS bacteriano;

4- O composto do chá verde, o EGCG, e o composto do cranberry, o AC-PACs possuem capacidade anti-inflamatória e efeito sinérgico quando em associação com o peptídeo LL-37 na regulação de citocinas inflamatórias frente à uma agressão por LPS bacteriano.

Levando-se em consideração as hipóteses, este trabalho teve como objetivo:

1- Avaliar a capacidade do extrato do chá verde e do EGCG sobre a produção e a expressão gênica de hBD-1 e hBD-2 pelas células epiteliais gengivais (B11), assim como avaliar a degradação das mesmas pelo *P. gingivalis* (capítulo 1).

2- Desenvolver um novo modelo em 3 dimensões de co-cultura de células epiteliais gengivais (OBA-9) e fibroblastos (HGF-1). Avaliar as interações celulares, assim como a resposta inflamatória deste modelo de co-cultura frente à uma agressão com LPS de *A. actinomycetemcomitans,* através da quantificação de IL-6 e IL-8 (capítulo 2).

3- Avaliar a capacidade anti-inflamatória e o efeito sinérgico dos peptídeos hBD-3 e LL-37 sobre a produção de citocinas, quimiocinas e fatores de crescimento em modelo de co-cultura celular estimulado com o LPS de *A. actinomycetemcomitans* (capítulo 2).

4- Avaliar a capacidade anti-inflamatória e o efeito sinérgico da associação do EGCG e do AC-PACs com o peptídeo LL-37 sobre a produção de citocinas, quimiocinas e fatores de

crescimento em modelo de co-cultura celular estimulado com o LPS de *A. actinomycetemcomitans* (capítulo 3).

Capítulo 1

Artigo publicado na Periodontal Research

Green tea extract and its major constituent epigallocatechin-3-gallate induce epithelial β-defensin secretion and prevent β-defensin degradation by *Porphyromonas gingivalis*

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Running title: Green tea polyphenols and epithelial β-defensins

ABSTRACT

Background and Objective: Antimicrobial peptides such as b-defensins secreted by gingival epithelial cells are thought to play a major role in preventing periodontal diseases. In the present study, we investigated the ability of green tea polyphenols to induce human b-defensin (hBD) secretion in gingival epithelial cells and to protect hBDs from proteolytic degradation by *Porphyromonas gingivalis*.

Material and Methods: Gingival epithelial cells were treated with various amounts (25-200 µg/mL) of green tea extract or epigallocatechin-3-gallate (EGCG). hBD-1 and hBD-2 secretion was measured using enzyme-linked immunosorbent assays (ELISA), and gene expression was quantified by real-time PCR. The treatments were also carried out in the presence of specific kinase inhibitors to identify the signaling pathways involved in hBD secretion. The ability of green tea extract and EGCG to prevent hBD degradation by proteases of *P. gingivalis* present in a bacterial culture supernatant was evaluated by ELISA.

Results: hBD-1 and hBD-2 secretion was dose-dependently upregulated following the stimulation of gingival epithelial cells with a green tea extract or EGCG. hBD gene expression in gingival epithelial cells treated with green tea polyphenols also increased. EGCG-induced hBD-1 and hBD-2 secretion appeared to involve extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase. Lastly, green tea extract and EGCG prevented the degradation of recombinant hBD-1 and hBD-2 by a culture supernatant of *P. gingivalis*.

Conclusion: Green tea extract and EGCG through their ability to induce hBD secretion by epithelial cells and to protect hBDs from proteolytic degradation by *P. gingivalis* have the potential to strengthen the epithelial antimicrobial barrier. Future clinical studies will indicate whether these polyphenols can represent a valuable therapeutic agent for treating/preventing periodontal diseases.

Keywords: epithelial cells, b-defensin, green tea, epigallocatechin gallate, periodontal disease, *Porphyromonas gingivalis*

INTRODUCTION

Periodontal diseases are destructive inflammatory disorders that affect the supporting tissues of the tooth and that result in attachment loss, periodontal pocket formation, alveolar bone resorption, and ultimately tooth loss. Periodontal diseases are complex multifactorial diseases the have two distinct but interconnected etiologic components: a limited group of Gram-negative bacteria, including *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*, colonizing the subgingival sites as biofilms (1), and an uncontrolled host inflammatory response to these periodontopathogens involving immune and mucosal cells (2).

The gingival epithelium is a stratified squamous tissue that acts as an interface between the external environment and the underlying periodontal connective tissue. It plays a key role in resisting periodontal infections by acting as a physical barrier against periodontopathogens and mounting an innate immune defense through the secretion of antimicrobial peptides, which include the human b-defensins (hBDs), more specifically hBD-1, hBD-2, and hBD-3 (3-5). These cationic antimicrobial peptides interact with the bacterial cell membrane, leading to pore formation and ultimately to the lysis of major periodontopathogens (5-9). Different lines of evidence have suggested that hBDs play a key role in maintaining periodontal health. It has been reported that mRNA expression levels of hBDs are higher in healthy gingival tissues as compared to the diseased ones (10-11). Furthermore, Brancatisano *et al.* (12) reported that there is a marked reduction in hBD-3 levels in the gingival crevicular fluid that parallels the severity of the disease in periodontitis patients. This reduction may be associated with the capacity of some periodontopathogenic bacteria such as *P. gingivalis* to down-regulate hBD expression by epithelial cells (13, 14) and/or to inactivate hBDs by proteolytic cleavage (15, 16). Moreover, young adults with morbus Kostmann syndrome, in which the expression of antimicrobial peptides is deficient due to an inherited defect, suffer from severe periodontal disease (17). Given the numerous beneficial properties of hBDs, including antimicrobial and anti-inflammatory activities

(4, 5) and the fact that decreased production of these antimicrobial peptides is associated with periodontal problems, conditions that increase their secretion by oral epithelial cells are likely to have a positive impact on periodontal health.

Tea is an aqueous aromatic infusion of dried leaves of the plant *Camellia sinensis*. It is the most popular beverage in the world after water. Tea contains numerous components, including amino acids, polysaccharides, alkaloids, vitamins, and polyphenols, mostly flavonoids (18). Green tea is composed of non-fermented leaves and has a high catechin content, including epigallocatechin-3-gallate (EGCG) (18). An emerging body of evidence indicates that green tea polyphenols may contribute to reducing the risk of cardiovascular disease and cancer and may have a variety of other beneficial effects on human health (19-20). The beneficial properties of green tea polyphenols have been associated with their anti-inflammatory, anti-oxidative, and anti-carcinogenic effects (19-20). While oral health benefits have been attributed to green tea throughout history, only a few scientific studies to validate these benefits have been carried out (21-24). A recent cross-sectional study in Japan showed that green tea consumption is associated with a decreased probability of tooth loss (21), while other animal and human studies have indicated that green tea consumption reduces dental caries (22). An epidemiological study on periodontal diseases published in 2009 showed that drinking one or more cups of green tea a day reduces the risk of periodontal disease (23). More specifically, green tea drinkers have healthier gums and teeth, and the beneficial effects are correlated with the amount of tea consumed (23). Moreover, a recent clinical study has shown that local delivery of green tea catechins into periodontal pockets enhances the efficacy of conventional periodontal treatments (24). Despite the studies suggesting that green tea polyphenols have a positive impact on periodontal health, it is still unclear how they exert these beneficial effects. In this study, we hypothesized that green tea polyphenols, more specifically EGCG, may contribute to periodontal health by inducing hBD secretion in gingival epithelial cells and preventing their degradation by the proteolytic periodontopathogen *P. gingivalis*.

MATERIALS AND METHODS

Green tea extract and epigallocatechin-3-gallate

The commercial green tea extract (Organic Herb Inc., Changsha, China) used in this study had a polyphenol content ≥98%, including 45% EGCG, according to the company's data sheet. A stock solution was freshly prepared by dissolving 20 mg of powder in 1 mL of sterile warm distilled water and filtering the solution through a 0.2-µm-pore membrane filter. EGCG (Sigma-Aldrich, Inc., St. Louis, MO, USA), the major catechin in green tea, was also dissolved in sterile distilled water at a concentration of 2 mg/mL and was filter sterilized.

Cultivation of oral epithelial cells

The immortalized human gingival epithelial cell line B11, which was kindly provided by Dr. Sabine Gröger (Department of Periodontology, Justus-Liebig-University, Germany) was previously characterized (25). The epithelial cells were cultured in K-SFM serum-free medium (Life Technologies Inc., Burlington, ON, Canada) containing insulin, epidermal growth factor, fibroblast growth factor, and 100 µg/mL of penicillin G/streptomycin at 37°C in a 5% CO_2 atmosphere until they reached confluence.

Stimulation of oral epithelial cells

The epithelial cells were harvested following a gentle trypsin treatment (5 min) (TrypLE™ Express; Life Technologies Inc.) at 37°C. The protease was then inactivated by adding 0.3 mg/mL of trypsin inhibitor, and the cells were harvested by centrifugation (500 *g* for 5 min), suspended in fresh medium, seeded in a 12-well microplate (1 mL/well, 1 x 10^6 cells/mL) (Sarstedt, Newton, NC, USA), and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion prior to stimulation. The cells were then stimulated with either green tea extract or EGCG (25-300 µg/mL) for 3 or 24 h at 37°C in a 5% CO_2 atmosphere. In order to investigate the

signaling pathways activated by green tea polyphenols that lead to hBD secretion, epithelial cells were also pre-treated for 2 h with protein kinase inhibitors (EMD Chemicals, Gibbstown, NJ, USA) (20 μM): SB203580 (p38 mitogen-activated kinase [p38 MAPK] inhibitor), and UO126 (extracellular-regulated kinase 1, 2 [ERK1/2] inhibitor) prior to EGCG (100 μg/mL) for 3 h (hBD mRNA expression) or 24 h (hBD secretion). Stock solutions (50 mM) of inhibitors were prepared in dimethyl sulfoxide (DMSO); a control assay with DMSO was included to confirm that this compound does not have any effect on cells at the final concentration tested (0.04%). Following the stimulation, the supernatants were collected and subjected to centrifugation (500 *g* for 5 min). The cells were washed once with PBS and mRNA was extracted (see below). All samples were stored at -80°C until used. Unstimulated epithelial cells were used as a control.

Determination of cell viability

A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany) using 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as the substrate was used to determine the effect of green tea extract and EGCG on gingival epithelial cell viability.

Enzyme-linked immunosorbent assay for secretion of b-defensins

Commercial enzyme-linked immunosorbent assay (ELISA) kits (PeproTech, Rocky Hill, NJ, USA) were used to quantify hBD-1 and hBD-2 concentrations in the cell-free supernatants according to the manufacturer's protocols. The absorbance at 405 nm was read using a microplate reader, with the wavelength correction set at 650 nm.

Real-time RT-PCR analysis of □-defensin gene expression

Total RNA was isolated from untreated and treated (green tea extract, EGCG ± protein kinase inhibitors) epithelial cells using commercial kits (RNeasy Plus mini, Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. The quality and quantity of the RNA in the

samples were determined using the Experion system and RNA StdSens analysis kits (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. The reverse transcription-polymerase chain reaction analysis (RT-PCR) was performed as follows. RNA from each sample (1 μg) was reverse-transcribed using Maloney murine leukemia virus reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA, USA). The reverse transcription conditions were 5 min at 70°C, 10 min at 25°C, 50 min at 37°C, and 15 min at 70°C. The amounts of mRNA transcript were measured using the Bio-Rad CFX96 RT-PCR detection system (Bio-Rad Laboratories). Triplicate reactions were prepared using 25 μL of a PCR mixture containing 12.5 μL of IQ SYBR Green Supermix (Bio-Rad Laboratories), 5 μL of cDNA, 1 μL of gene-specific primer (hBD-1, hBD-2), and 6.5 μL of RNase- and DNase-free water. The sequences of the primers (Life Technologies Inc.) used for the RT-PCR are listed in Table 1. The samples were amplified using a Bio-Rad MyCycler^{TM} thermal cycler (Bio-Rad Laboratories). The amplification conditions for the hBD-1 and hBD-2 primers were 95°C for 3 min followed by 40 cycles at 95°C for 10 s, 56.9°C for 10 s, and 72°C for 30 s. To validate the specificity of each primer pair, temperature curve analyses were performed. The glycerate-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for data normalization. The amplification conditions for GAPDH primers (Table 1) were 10 min at 95°C followed of 40 cycles of 30 sec at 95°C, and 30 sec at 60°C.

b-defensin degradation by *P. gingivalis* culture supernatant

The ability of green tea extract and EGCG to protect hBDs against degradation by proteases of *P. gingivalis* present in a bacterial culture supernatant was investigated as follows. Recombinant hBD-1 (1000 pg/mL) or hBD-2 (2000 pg/mL) (PeproTech) was incubated with an overnight culture supernatant (1:20 dilution in PBS) of the reference strain *P. gingivalis* ATCC 33277 in the absence or presence of green tea extract (100 μg/mL) or EGCG (50 μg/mL), which was added 10 min before the hBDs. A culture supernatant treated at 100ºC for 5 min was used as negative

control. The above mixtures were incubated for 4 h at 37°C under anaerobic conditions. Following the incubation, the supernatants were collected and were stored at -80°C until residual hBDs were quantified by ELISA as described above.

Statistical analysis

All assays were performed in triplicate and the means ± standard deviations (SD) were calculated. Experiments were carried for a minimum of three times to ensure reproducibility, and data from a representative experiment are presented. Differences between means were analyzed for statistical significance using a one-way analysis of variance (ANOVA).

RESULTS

Cytotoxicity of green tea extract and EGCG

We first evaluated the effect of green tea extract on the viability of the B11 epithelial cell line. As reported in Table 2, the green tea extract did not have a significant cytotoxic effect at concentrations up to 200 μg/mL. However, a decrease in cell viability was observed following a treatment with 300 μg/mL of the green tea extract. To avoid any deleterious effects on epithelial cells, the treatments were performed with ≤ 200 μg/mL of green tea extract.

Effect of green tea extract and EGCG on b-defensin secretion

The ability of the green tea extract to induce the secretion of hBD-1 and hBD-2 by gingival epithelial cells was determined by ELISA. Figure 1 shows that the secretion of both hBDs was dose-dependently upregulated following the stimulation of the epithelial cells with the green tea extract. In the presence of 200 μg/mL of green tea extract, the epithelial cells secreted 1603 pg/mL of hBD-1 and 928 pg/mL of hBD-2. The B11 epithelial cell line used in this study appears not to have the ability to secrete hBD-3 (data not shown).

Given the high EGCG content of the green tea extract, we postulated that this polyphenol may be the bioactive compound involved in upregulating hBD secretion. Non-cytotoxic concentrations of EGCG were first determined (Table 2). EGCG was used at concentrations ≤ 100 μg/mL to avoid a deleterious effect on the gingival epithelial cells. Figure 2 shows that EGCG induced the secretion of hBD-1 and hBD-2 in a dose-dependent manner. More specifically, EGCG at 100 μg/mL induced the secretion of 1800 pg/mL of hBD-1 and 1009 pg/mL of hBD-2.

Effect of green tea extract and EGCG on b-defensin gene expression

To confirm the ELISA results, we measured the expression of hBD-1 and hBD-2 mRNA by epithelial cells treated with green tea extract and EGCG. As shown in Figure 3, the green tea extract dose-dependently increased hBD-2 mRNA levels and, to a lesser extent, hBD-1 mRNA levels. A dose-dependent response was also observed for EGCG (Figure 4). More specifically, 100 μg/mL of EGCG increased hBD-1 and hBD-2 mRNA levels 2.7- and 3.3-fold, respectively (Figure 4).

Effect of kinase inhibitors on EGCG-induced b-defensin secretion and gene expression

To investigate the EGCG-activated signaling pathways that modulate hBD-1 and hBD-2 secretion and mRNA expression by epithelial cells, the stimulations with EGCG were performed in the presence of selective p38 MAPK and ERK1/2 inhibitors at a concentrations (20 μM) that showed no cytotoxicity (data not shown). As shown in Table 3, in the presence of 100 μg/mL of EGCG, the selective inhibitor of p38 MAPK reduced hBD-1 and hBD-2 secretion by 68% and 69%, respectively, while the selective inhibitor of ERK1/2 reduced hBD-1 secretion by 34%, but had no effect on hBD-2 secretion. When used in the absence of EGCG, none of the kinase inhibitor had an effect on hBD secretion (data not shown). Both kinase inhibitors almost completely inhibited the expression of hBD-1 and hBD-2 mRNA (Figure 5).

Effect of green tea extract and EGCG on b-defensin degradation by *P. gingivalis* proteases

We then investigated the ability of green tea extract and EGCG to protect hBD-1 and hBD-2 against proteolytic degradation by a *P. gingivalis* culture supernatant. As shown in Table 4, the culture supernatant of *P. gingivalis* almost completely degraded both hBDs following a 4-h incubation. However, 50 μg/mL of EGCG prevented the degradation of hBD-1 and hBD-2 by *P. gingivalis* proteases. To a lesser extent, green tea extract (100 μg/mL) also protected hBDs from proteolytic degradation.

DISCUSSION

Given the high prevalence of periodontal diseases in the general population, their negative impact on daily quality of life, and the fact that emerging evidence supports a link between these diseases and systemic health problems (preterm baby delivery, diabetes, cardiovascular diseases, etc) (26, 27), studies on novel preventive and therapeutic strategies for treating periodontal diseases are highly relevant. Previous reports have suggested that green tea contains bioactive molecules that can promote periodontal health (21, 23, 24). In the present study, we investigated the ability of a green tea extract and its main polyphenolic constituent EGCG to induce the secretion by gingival epithelial cells of antimicrobial hBDs and to prevent their degradation by *P. gingivalis*.

We first showed that a green tea extract increased the secretion of hBD-1 and hBD-2 by epithelial cells. Since many of the health beneficial effects associated with green tea have been attributed to EGCG (18-20) and that it represents the major component of the green tea extract used in this study, this catechin was used to stimulate the epithelial cells. EGCG was found to dose-dependently induce the secretion of hBD-1 and hBD-2. In our experimental protocol, gingival epithelial cells were treated with green tea extract or EGCG for 24 h; further studies should investigate how epithelial cells respond to shorter exposure times to green tea polyphenols. Real-time PCR analysis showed that EGCG also increased the expression of the hBD-1 and hBD-2 mRNA. Given that the above observations were obtained using an immortalized epithelial cell line, studies in progress are using primary gingival epithelial cells isolated from periodontitis and healthy patients. Although our data suggest that EGCG is the bioactive ingredient in the green tea extract, ones should not exclude the possibility that additional components in the extract can also induce the secretion of hBD-1 and hBD-2. To the best of our knowledge, this is the first report on the capacity of polyphenols to trigger the upregulation of hBDs in epithelial cells. However, in a

recent study, McMahon et al. (28) reported on the capacity of the active form of vitamin D, $1,25(OH)_2D_3$, to induce the expression of the antimicrobial peptide cathelicidin LL-37 in human gingival epithelial cells.

The ability of green tea extract and EGCG to upregulate the secretion of hBDs may have several positive impacts on periodontal health. First, it is well known that hBDs are active against the major periodontopathogens (4-7) and may contribute to reducing the colonization of subgingival sites and the invasion of periodontal tissues by these pathogens. Second, hBDs, including hBD-2, attenuate cytokine secretion by lipopolysaccharide (LPS)-stimulated epithelial cells and macrophages (29-30), thus reducing gingival inflammation. Third, hBD-2 potentiates wound healing by increasing cell migration and proliferation (31, 32).

In a previous study, we reported that tea extracts (green, white, black, and oolong) can inhibit the growth of *P. gingivalis* and reduce the ability of this periodontopathogen to adhere to epithelial cells and degrade type I collagen (33). These extracts also reduced IL-6, IL-8, and CCL-5 production by LPS-stimulated gingival epithelial cells (33). Results from the present study provided further support for the beneficial effect of green tea on periodontal health. EGCG, a powerful antioxidant molecule (34) with the ability to protect cells against damage mediated by free radicals, was identified as one of the bioactive molecules involved in the upregulation of hBD secretion and expression. EGCG is a major component of green tea. Wu and Wei (35) reported that a cup of green tea (2.5 g of green tea leaves/200 mL of water) may contain up to 90 mg of EGCG. Interestingly, previous studies have shown that EGCG may also be a potential anti-cariogenic agent because of its capacity to inhibit the growth, biofilm formation, and acid production of *Streptococcus mutans* (36-38).

We used specific protein kinase inhibitors to show that EGCG-induced hBD-1 and hBD-2 secretion involves members of the mitogen-activated protein kinase (MAPK) family. On the one hand, ERK1/2 and p38 MAPK inhibitors were effective in inhibiting hBD-1 protein and mRNA expression. On the other hand, the p38 MAPK inhibitor prevented secretion and mRNA expression of hBD-2 while ERK1/2 inhibitor inhibited mRNA expression of hBD-2 but had no effect on hBD-2 secretion. This latter observation may be related to the fact that mRNA expression was monitored following a 3-h incubation while protein secretion was monitored following a 24-h incubation. It can be hypothesized that the ERK1/2 inhibitor is efficient initially but cannot maintain inhibition over a long period of incubation. Moreover, because hBDs may have an intracellular location in mammalian cells, hBD secretion may not be directly related to hBD mRNA expression. Therefore, we may observe an inhibitory effect of a specific protein kinase inhibitor on mRNA expression that does not correlate with an inhibition of protein secretion. Quantification of cellular hBDs may bring evidence to confirm this hypothesis. Recently, a natural sugar extract from the *Avocado gratissima* fruit has been shown to upregulate hBD-2 expression in skin epithelia (39, 40). In agreement with our results, Paoletti et al. (40) provided evidence for a role for ERK/MAPK signaling pathways in hBD secretion. The p38 MAPK pathway has also been shown to be involved in the regulation of hBD-2 expression in epithelial cells treated with *Fusobacterium nucleatum* (41) and nicotine (42). Studies in progress in our laboratory are aimed to characterize in more details the EGCG-activated ERK/MAPK signaling pathways leading to hBD expression and to identify the epithelial cell surface receptors involved.

Contradictory results exist in the literature regarding the expression and levels of hBDs in patients with periodontitis (10-12, 43) and this may be related to the specific microflora found in diseased periodontal sites. Previous studies have reported that *P. gingivalis*, but not other periodontopathogens such as *T. denticola* (4) can induce b-defensin expression in gingival

epithelial cells (45, 46). *P. gingivalis* produces proteinases called gingipains that can degrade a broad array of tissue and plasma proteins (47). In the present study, we showed that secreted proteases of *P. gingivalis* are able to degrade hBD-1 and hBD-2. The inactivation of these innate epithelium-derived antimicrobial peptides may have an impact on the host protection capacity against periodontal infections. However, we showed that the green tea extract and EGCG can counteract the above-mentioned deleterious effects associated with *P. gingivalis* by inhibiting hBD degradation. This may contribute to maintaining an effective level of hBDs in subgingival sites colonized by proteolytic periodontopathogens. The ability of EGCG to inhibit matrix metalloproteinases, which are well known to contribute to degradation of periodontal tissue proteins (48), has been previously reported (49) and further support the protective role of green tea polyphenols against periodontal diseases. In a previous study by Madhan et al. (50), the ability of EGCG to inhibit proteolytic enzymes has been related to a change in the secondary structure of the enzymes through hydrogen bonding and hydrophobic interactions.

The results from the present study will serve as the foundation for human clinical trials aimed at demonstrating that the daily intake of green tea or the use of oral hygiene products (mouthrinses, chewing gums) or slow periodontal release devices (to be inserted in diseased periodontal sites) containing green tea polyphenols may be an economic and safe way to maintain the periodontal health of the general population. The results of the clinical trials may lead to the development of applications for the treatment of non-oral problems since other epithelial cells (intestinal, urinary tract, etc.) may respond to green tea stimulation by secreting hBDs.

CONCLUSION

By inducing hBD secretion by gingival epithelial cells and preventing the proteolytic degradation of these antimicrobial peptides, green tea polyphenols, especially EGCG, hold promise as a

therapeutic agent for treating/preventing periodontal diseases by strengthening the epithelial antimicrobial barrier.

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Table 1. Primers used for the real-time PCR analysis.

Genes	**Primer sequences**	**Product size (bp)**
GAPDH	Sense: 5-GGT ATC GTC GAA GGA CTC ATG AC-3’ Antisense: 5’-ATG CCA GTG AGC TTC CCG TTC AGC-3’	180
hBD-1	Sense: 5’-GCC TCT CCC CAG TTC CTG AA-3’ Antisense: 5’-GCA GAG AGT AAA CAG CAG AAG GTA-3’	82
hBD-2	Sense: 5’-TGT GGT CTC CCT GGA ACA AAA T-3’ Antisense: 5’-GTC GCA CGT CTC TGA TGA GG-3’	105

Table 2. Effect of a green tea extract and EGCG on the viability of gingival epithelial cells.

Concentration (μg/mL)	% cell viability[a]	
	Green tea extract	EGCG
300	72 ± 11[b]	61 ± 9[b]
200	95 ± 7	75 ± 9[b]
100	105 ± 9	91 ± 8
50	98 ± 11	95 ± 2
25	103 ± 4	98 ± 7
0	100	100

[a] Means ± SD of triplicate assays.

[b] Significantly different ($P < 0.01$) from untreated control cells as determined using ANOVA.

Table 3. Effect of kinase inhibitors on the secretion of β-defensins by gingival epithelial cells treated with EGCG.

Inhibitor	Kinase specificity	Relative secretion (%)	
		hBD-1	hBD-2
None	_	100	100
SB203580	p38 MAPK	32.4*	31.5**
UO126	ERK1/2	66.2*	108.1

Significantly different (*, $P < 0.01$; **, $P < 0.001$) from control cells (without inhibitor) as determined using ANOVA.

Table 4. Effect of green tea extract and EGCG on the proteolytic degradation of hBD-1 and hBD-2 by a culture supernatant of *P. gingivalis*.

Conditions	Amounts of hBDs (pg/mL)[a]	
	hBD-1	hBD-2
Non-inoculated culture medium (control)	912 ± 28	1803 ± 71
P. gingivalis culture supernatant	95 ± 13[b]	114 ± 21[b]
P. gingivalis culture supernatant + green tea extract (100 μg/mL)	772 ± 18	1395 ± 20
P. gingivalis culture supernatant + EGCG (50 μg/mL)	880 ± 34	1704 ± 76

[a]Means ± SD of triplicate assays.

[b]Significantly different ($P < 0.001$) from control assay (non-inoculated culture medium) as determined using ANOVA.

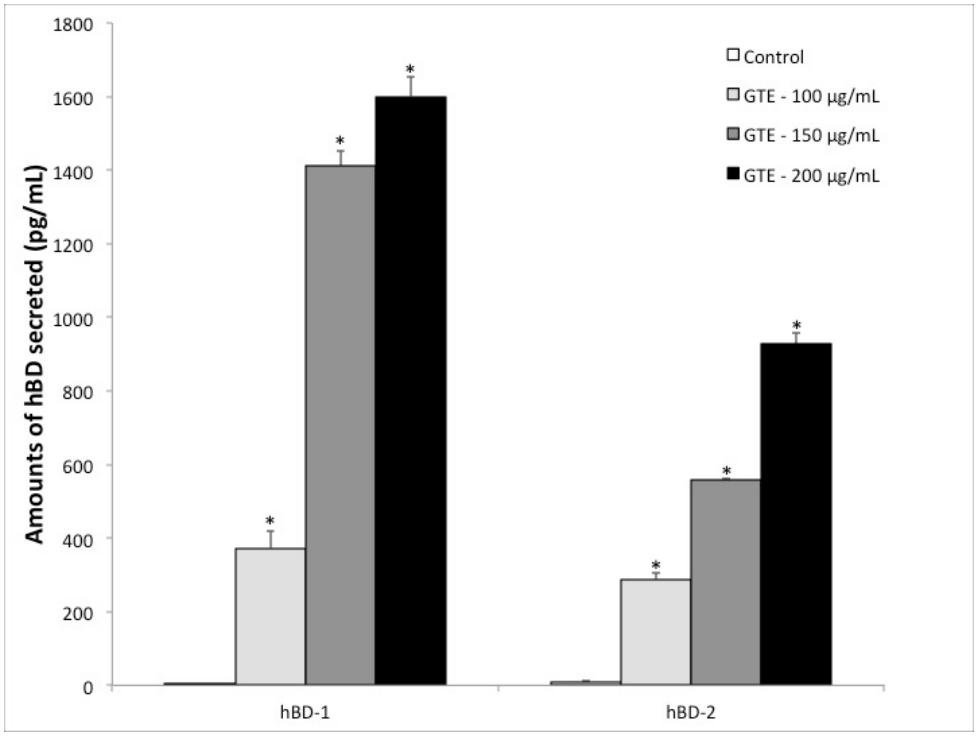

Figure 1. Effect of a green tea extract (GTE) on the secretion of hBD-1 and hBD-2 by gingival epithelial cells. hBDs were quantified by ELISA following a 24-h stimulation of epithelial cells. The means ± SD was calculated from triplicate assays. Significantly different (*, $P < 0.001$) from control untreated epithelial cells as determined using a ANOVA.

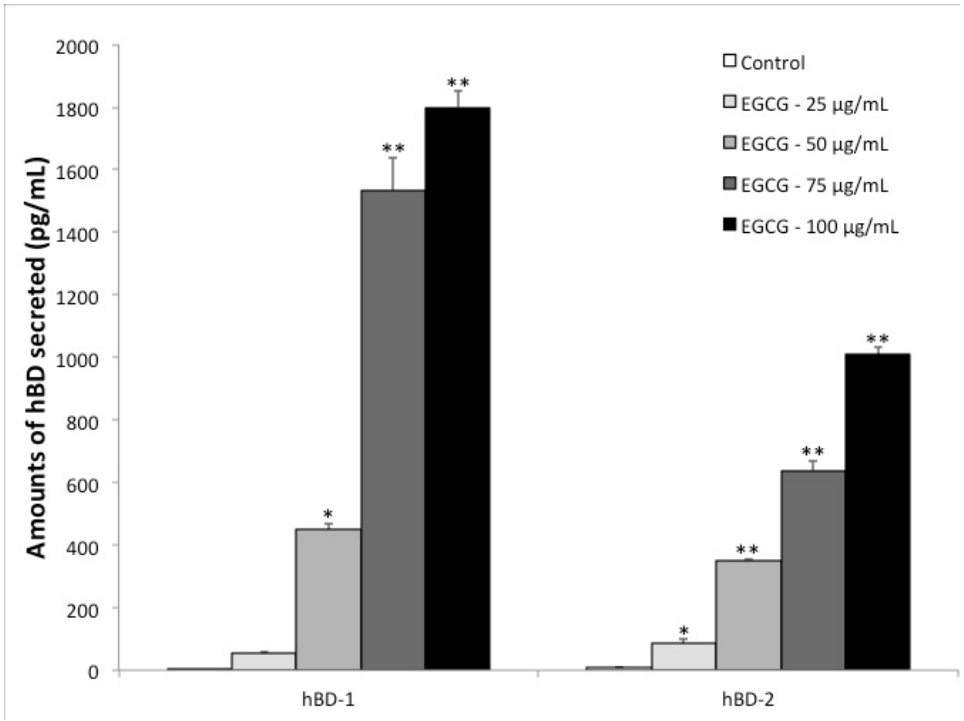

Figure 2. Effect of EGCG on the secretion of hBD-1 and hBD-2 by gingival epithelial cells. hBDs were quantified by ELISA following a 24-h stimulation of epithelial cells. The means ± SD was calculated from triplicate assays. Significantly different (*, $P < 0.01$; **, $P < 0.001$) from control untreated epithelial cells as determined using ANOVA.

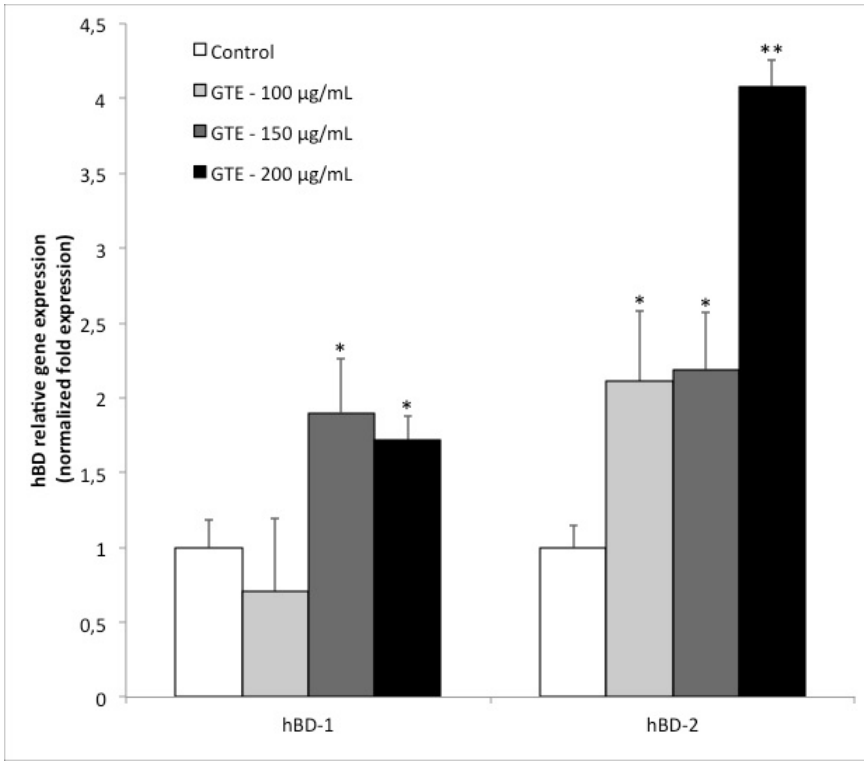

Figure 3. Effect of a green tea extract (GTE) on hBD-1 and hBD-2 mRNA expression by gingival epithelial cells. hBD mRNA expression was quantified by reat-time RT-PCR following a 3-h stimulation of epithelial cells. The means ± SD was calculated from triplicate assays. Significantly different (*, $P < 0.05$; **, $P < 0.01$) from control untreated epithelial cells as determined using ANOVA.

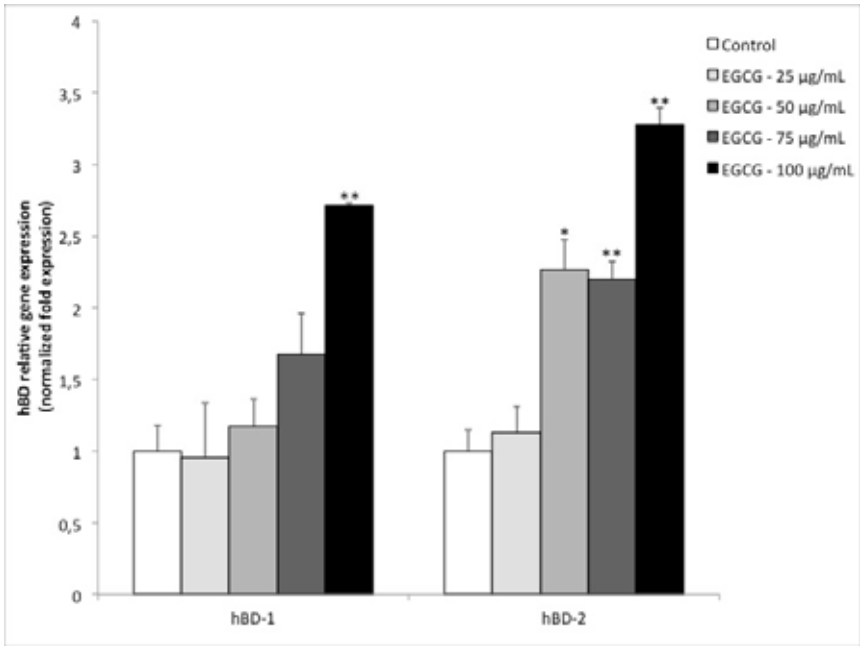

Figure 4. Effect of EGCG on hBD-1 and hBD-2 mRNA expression by gingival epithelial cells. hBD mRNA expression was quantified by real-time RT-PCR following a 3-h stimulation of epithelial cells. The means ± SD was calculated from triplicate assays. Significantly different (*, $P < 0.05$; **, $P < 0.01$) from control untreated epithelial cells as determined using ANOVA.

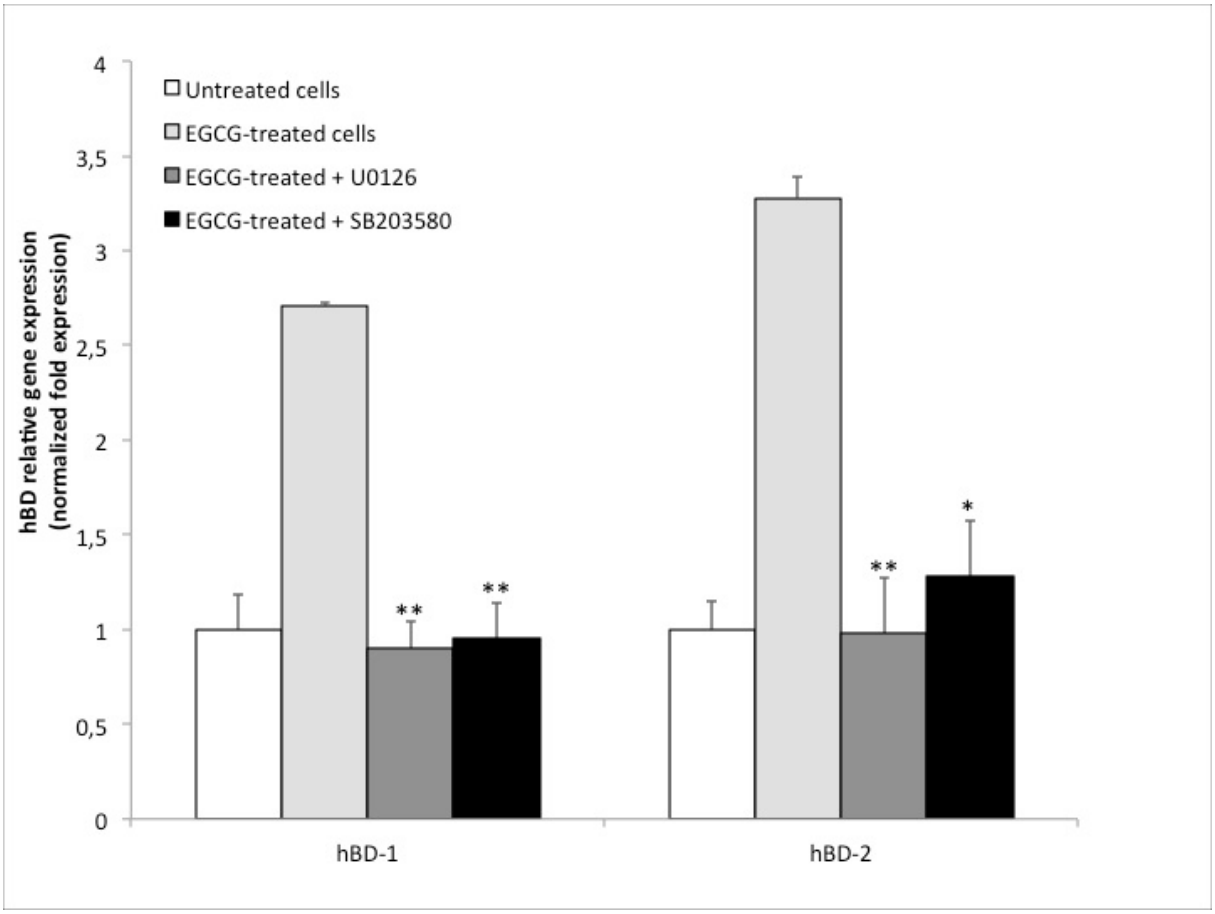

Figure 5: Effect of UO126 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor) on EGCG-induced hBD-1 and hBD-2 mRNA expression by gingival epithelial cells. Epithelial cells were pre-treated (2 h) with the kinase inhibitors prior to be stimulated with EGCG (100 µg/mL) for 3 h and to quantify hBD mRNA expression by real-time RT-PCR. The means ± SD was calculated from triplicate assays. Significantly different (*, $P < 0.05$, **, $P < 0.01$) from control EGCG-treated epithelial cells (no kinase inhibitor) as determined using ANOVA.

Capítulo 2

Artigo submetido – Peptides

Anexo 1 (carta de envio)

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

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Abstract

With the spread of antibiotic resistance in bacterial pathogens, antimicrobial peptides that have antibacterial activity and that can also modulate the immune response may be a novel approach for effectively controlling periodontal infections. In the present study, we used a three-dimensional (3D) co-culture model of gingival epithelial cells and fibroblasts stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) to investigate the anti-inflammatory properties of human beta-defensin-3 (hBD-3) and cathelicidin (LL-37) and to determine whether these peptides can act in synergy. A 3D co-culture model composed of gingival fibroblasts embedded in a collagen matrix overlaid with gingival epithelial cells had a synergistic effect with respect to the secretion of IL-6 and IL-8 in response to LPS stimulation compared to fibroblasts and epithelial cells alone. The 3D co-culture model was stimulated with non-cytotoxic concentrations of hBD-3 (10 and 20 μM) and LL-37 (0.1 and 0.2 μM) individually and in combination in the presence of *A. actinomycetemcomitans* LPS. A multiplex ELISA assay was used to quantify the secretion of 41 different cytokines. hBD-3 and LL-37 acted in synergy to reduce the secretion of GRO-α, G-CSF, IP-10, IL-6, and MCP-1, but only had an additive effect on reducing the secretion of IL-8 in response to *A. actinomycetemcomitans* LPS stimulation. The present study showed that hBD-3 acted in synergy with LL-37 to reduce the secretion of cytokines by an LPS-stimulated 3D model of gingival mucosa. This combination of antimicrobial peptides thus shows promising potential as an adjunctive therapy for treating inflammatory periodontitis.

Keywords: beta-defensin, cathelicidin, cytokine, epithelial cell, fibroblasts, periodontal disease

Introduction

Periodontitis is a multifactorial chronic inflammatory disease of polymicrobial origin that causes the destruction of the tooth-supporting tissues, including the periodontal ligament and alveolar bone [1]. A limited number of Gram-negative, mostly anaerobic bacteria that colonize the subgingival area and that activate the host immune response have been associated with this disease [2]. More specifically, *Aggregatibacter actinomycetemcomitans* is considered to be a key etiological agent of aggressive periodontitis [3]. The lipopolysaccharide (LPS) of *A. actinomycetemcomitans* is a major virulence factor [4]. This component of the outer membrane can promote adhesion to oral cells and can activate the host immune response, resulting in the secretion of large amounts of pro-inflammatory cytokines, including interleukin-6 (IL-6) and interleukin-8 (IL-8), that can contribute to the destruction of periodontal tissues [4–6].

Epithelial cells and fibroblasts are the predominant cells of periodontal tissues and serve as a first line of defense against periodontopathogens. They act as a mechanical barrier against bacterial invasion in addition to secreting different classes of inflammatory mediators such as cytokines, chemokines, and tissue-destructive enzymes in response to pathogen stimulation. When the immune and inflammatory responses do not stop the progression of an infection, uncontrolled secretion of cytokines occurs, leading to chronic inflammation and periodontal tissue destruction [7]. Higher levels of IL-8 and monocyte chemo-attractant protein 1 (MCP-1) have been found in gingival crevicular fluid (GCF) from periodontitis sites than in GCF from healthy control sites, while their levels decrease after periodontal treatments [8–12].

Traditional scaling and root planing remain the “gold standard” for the treatment of periodontitis. However, some patients do not respond adequately to the conventional

treatment and require adjunctive antimicrobials. Given that many bacteria have developed resistance to antibiotics, new strategies need to be developed for adjunctive therapies [13].

Antimicrobial peptides (AMPs) are small cationic molecules of the innate immune response with a broad activity spectrum against pathogens, including those associated with periodontitis [14]. Gingival epithelial cells have been reported to secrete several AMPs either constitutively or in response to an infection [15,16]. LL-37 and human β-defensin (hBD-3) are the most important AMPs found in humans. CAP18 is the only member of the cathelicidin family found in humans. The C-terminal of CAP18 is proteolytically cleaved to generate LL-37, a 37-amino-acid peptide beginning with two leucine residues [17,18]. hBD-3 is an important defensin in the oral cavity and is expressed in response to bacterial invasion [19,20]. Both hBD-3 and LL-37 have a broad activity spectrum and have been detected in GCF and saliva [21–24]. Some studies have reported a marked reduction in the amounts of hBD-3 and LL-37 in gingival crevicular fluid during periodontitis, which could be related to the ability of certain periodontopathogens to proteolytically inactivate the peptides or down-regulate their expression [25–27].

hBD-3 and LL-37 can penetrate the periplasmic space of Gram-negative bacteria, forming pores and lysing the bacteria [14,19,28–32]. In addition to being antimicrobial peptides, both modulate the immune response. hBD-3 and LL-37 can neutralize the inflammatory potential of LPS by binding directly to LPS or by preventing the binding of LPS to host cell receptors, thus blocking the cell signaling pathway triggered by TLR ligands [14,33,34]. Walter et al. [35] showed that LL-37 reduces cytokine secretion by LPS-stimulated human whole blood and proposed that this peptide should be considered for use in adjunctive periodontal treatments. By acting on the two etiological factors of periodontitis, i.e., periodontopathogens

and the inflammatory response, hBD-3 and LL-37 are very attractive candidates for adjunctive periodontal treatments.

hBD-3 and LL-37 may also work in association with other antimicrobial agents to enhance their antibacterial activities [36–38]. Chen et al. [39] showed that hBD-3 and LL-37 worked in synergy against *Staphylococcus aureus.* This may be related to the fact that hBD-3 and LL-37 are able to bind to different cell receptors. hBD-3 and LL-37 may also work synergistically to enhance their individual anti-inflammatory activities. However, to the best of our knowledge, there are no reports in the literature indicating that hBD-3 and LL-37 can act in synergy to reduce the LPS-induced inflammatory response of host cells.

So considering the beneficial antimicrobial and anti-inflammatory effect of the peptides hBD-3 and LL-37 and the possibility that some bacteria could inactivated those peptides (25-27), we hypothesized that if those peptides in association could have an anti-inflammatory synergistic effect to reduce cytokines secretion in response to an LPS infection

Previous studies on the anti-inflammatory properties of AMPs with respect to periodontal disease have used cell lines in monoculture [14,35,40], although we hypothesized that if the two most important cell lines present in the periodontal area (epithelial cells and fibroblasts) in association, could be shown an synergistic cytokine secretion in response to a LPS infection. In the present study, we used an in vitro three-dimensional (3D) co-culture model of gingival epithelial cells and fibroblasts to mimic the interaction between these two major cell types of the periodontium and analyzes if this two types of cell lines could shown an synergistic IL-6 and IL-8 secretion in response to *A. actinomycetemcomitans* LPS . We stimulated the 3D co-culture model with *A. actinomycetemcomitans* LPS to evaluate the effect

of cell interactions on cytokine secretion and to investigate the synergistic anti-inflammatory activities of hBD-3 and LL-37.

Materials and methods

Reagents and LPS preparation

The synthetic hBD-3 (H-GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCC-RRKK-OH) and LL-37 (H-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-OH) peptides were from Biomatik (Cambridge, ON, Canada). They were dissolved in sterile UltraPure™ DNase/RNase-free distilled water (Life Technologies Inc., Burlington, ON, Canada) at a concentration of 1 mM and were stored at –20°C until used. *A. actinomycetemcomitans* (ATCC 29522) LPS was isolated as described previously [41]. Stock solutions (1 mg/mL) prepared in sterile distilled water were stored at -20°C until used.

Cultivation of gingival epithelial cells and fibroblasts

The immortalized human gingival epithelial cell line OBA-9 [42], which was kindly provided by M. Mayer (Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil), was cultured in keratinocyte serum-free medium (K-SFM; Life Technologies Inc.) containing insulin, epidermal growth factor, fibroblast growth factor, and 100 µg/mL of penicillin G-streptomycin. The primary human gingival fibroblast cell line HGF-1 (ATCC® CRL-2014) was from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA), 10% heat-inactivated fetal bovine serum (FBS), and 100 µg/mL of penicillin G-streptomycin. Both cell lines were incubated at 37°C in a 5% CO_2 atmosphere until they reached confluence.

Preparation of the three-dimensional (3D) co-culture model

A 3D co-culture model composed of gingival fibroblasts embedded in a collagen matrix

overlaid with gingival epithelial cells was prepared according to the protocol described by Gursoy et al. [43], with slight modifications, we also performed some pilot studies to determine the incubation time of the 3D co-culture model to get a fibroblasts and gingival epithelial cells differentiation and a confluence around 90%. So, a commercial bovine type I collagen solution (95-98%; PureCol, Advanced BioMatrix, Tucson, AZ, USA) was mixed with DMEM (10X) (Sigma-Aldrich Canada, Oakville, ON, Canada) on ice to obtain a final collagen concentration of 76-78%. The pH was adjusted to 7. Confluent HGF-1 cells were detached by gentle trypsinization (0.05% trypsin-EDTA; Gibco-BRL, Grand Island, NY, USA). The trypsin was then inactivated by adding DMEM + 10% FBS. The cells were harvested by centrifugation (500 x g for 5 min) and were suspended at a density of 5 x 10^5 cells/mL in the collagen solution described above. The collagen cell suspension was placed in the wells of 6-well tissue culture plates (2 mL/well; 2.5-mm-thick) (Sarstedt, Newton, NC, USA). The collagen gel was allowed to solidify for 2 h at 37°C under aerobic conditions, and the plates were then incubated for a further 10 h at 37°C in a 5% CO_2 atmosphere. The OBA-9 cells were detached by gentle trypsinization (5 min) (TrypLE™ Express; Life Technologies Inc.) at 37°C. The trypsin was then inactivated by adding 0.3 mg/mL of trypsin inhibitor, and the cells were harvested by centrifugation (500 x g for 5 min) and suspended in fresh K-SFM medium. Aliquots (2 mL) of OBA-9 cells were seeded on top of the collagen-fibroblast gels at a density of 1 x 10^6 cell/mL. The 3D co-culture model (Figure 1) was incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion prior to stimulation.

Comparative analysis of LPS-induced IL-6 and IL-8 secretion by the 3D co-culture model and the individual cell lines

The 3D co-culture model was stimulated with *A. actinomycetemcomitans* LPS (1 µg/mL) for 24 h at 37°C in a 5% CO_2 atmosphere. Unstimulated cells (individually and in co-culture)

were used as controls. The supernatants were collected, centrifuged (3000 x g for 10 min at 4°C), and then stored at -20°C until used for the IL-6 and IL-8 assays. After a 24-h incubation, the co-culture model, the collagen-fibroblast gel, and the epithelial cells were visualized by inverted phase-light microscopy. Commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscence, Inc., San Diego, CA, USA) were used to quantify the IL-6 and IL-8 concentrations in the culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm (A_{450}) was recorded using a microplate reader with the wavelength correction set at 570 nm. The rated sensitivities of the commercial ELISA kits

Stimulation of the 3D co-culture model

The 3D co-culture model was pre-treated for 2 h with hBD-3 (10 and 20 μM) or LL-37 (0.1 and 0.2 μM), or both, prior to being stimulated with *A. actinomycetemcomitans* LPS (1 μg/mL) for 24 h at 37°C in a 5% CO_2 atmosphere. Co-cultures not pre-treated with hBD-3 or LL-37 and not stimulated with LPS were used as controls. The supernatants were collected, centrifuged (1000 x g for 5 min at 4°C), and stored at –20°C until used.

We selected those concentrations of each peptide mentioned above, based on previous studies (data not shown) in which we stimulated 3D co-culture model with the peptides (hBD-3 and LL-37) individually at 0.01, 0.05, 0.1, 0.2, 1, 5, 10, 20 and 40 μM, 2 hours prior to the stimulation with *A. actinomycetemcomitans* LPS (1 μg/ml). After 24 hours of incubation, the supernant were collected, centrifugue and we performed an ELISA sanduiche technique to quantify IL-6 and IL-8 present in the supernant sample. Based on those results we concluded that LL-37 at 0.1 and 0.2 and hBD3 at 10 and 20 μM showed an slight anti-inflammatory effect, so we choose those concentration to tested in association.

After choose those concentration of hBD-3 and LL-37 to tested the anti-inflammatory

synergistic effect, we would like to see if the peptides alone without LPS stimulation could be able to increase the IL-6 and IL-8 secretion by the 3D co-culture model. So we performed an experiment in which we stimulated the 3D co-culture model with the peptides individually without LPS stimulation,after 24 hours of incubation, the supernant were collected, centrifugue and we performed an ELISA sanduiche technique to quantify IL-6 and IL-8 present in the supernant sample.. We didn't observed any statistically

Determination of the viability of the OBA-9 and HGF-1 cell lines

We determined the effect of hBD-3, LL-37, and *A. actinomycetemcomitans* LPS, individually and in combination, on the viability of the OBA-9 and HGF-1 cells. Briefly, HGF-1 and OBA-9 cells (1 x 10^4 cells/well) were seeded in the wells of a 96-well microplate (0.1 mL/well) (Sarstedt) and were incubated for 4 h at 37°C in a 5% CO_2 atmosphere to allow cell adhesion. The culture medium was then aspirated, and the cells were pre-treated for 2 h with hBD-3 (5, 10, 20, 40 µM) and/or LL-37 (0.05, 0.1, 0.2, 0.5, 1, 5 µM) prior to adding 1 µg/mL of *A. actinomycetemcomitans* LPS. The cells were incubated for an additional 24 h at 37°C in a 5% CO_2 atmosphere. A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany) using 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as the substrate was performed according to the manufacturer's protocol. Untreated control cells were assigned a value of 100%, and all the other conditions were compared to the control. Results are expressed as means ± standard deviations of duplicate assays from two independent experiments.

Determination of cytokine secretion using multiplex ELISA assays

Samples of the 3D co-culture model subjected to the various treatments were sent to Eve Technologies (Calgary, AB, Canada, http://www.evetechnologies.com) for multiplex ELISA

analyses. Eve Technologies uses the Bio-Plex Suspension Array System to quantify 41 different cytokines, chemokines, and growth factors (Human 41-Plex Discovery Assay): epidermal growth factor (EGF), C-C motif chemokine 11 (Eotaxin-1), basic fibroblast growth factor (FGF-2), FMS-like tyrosine kinase 3 ligand (Flt3l), chemokine (C-X3-C motif) ligand 1 (Fractalkine), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), CXC-chemokine ligand 1 (GRO-α), interferon alpha 2 (IFN-α2), interferon gamma (IFNγ), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-12B (IL-12B), interleukin-12 (p70) [IL-12 (p70)], interleukin-13 (IL-13), interleukin-15 (IL-15), interleukin 17A (IL-17A), interferon-γ inducible protein 10 (IP-10), monocyte chemo-attractant protein 1 (MCP-1), monocyte-specific chemokine 3 (MCP-3), C-C motif chemokine 22 (MDC), macrophage inflammatory protein 1 α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), platelet-derived growth factor AA (PDGF-AA), platelet-derived growth factor AB/BB (PDGF-AB/BB), regulated on activation, normal T cell expressed and secreted (RANTES), soluble CD40 ligand (sCD40L), transforming growth factor alpha (TGF-α), tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), and vascular endothelial growth factor A (VEGF-A).

Data analysis

To determine the synergistic inhibitory effect of hBD-3 and LL-37 on cytokine secretion following stimulation of the 3D co-culture model with *A. actinomycetemcomitans* LPS, the

sums of the inhibition values of each peptide were compared with the values of both peptides used in combination. Experiments were carried out a minimum of three times to ensure reproducibility. The means ± SD from a representative experiment are presented. Differences between the means were analyzed for statistical significance using a one-way ANOVA. Statistical significance was set at $p < 0.05$.

Results

The HGF-1 gingival fibroblast cells (Figure 2A), OBA-9 gingival epithelial cells (Figure 2B), and the 3D co-culture model composed of both cell types were observed by light microscopy. Given the density and close proximity of epithelial cells and fibroblasts in the 3D co-culture model, several interactions between the two cells types are likely to occur.

Pilot experiments showed that the treatment of the HGF-1 and OBA-9 cells with 1 μ g/mL of *A. actinomycetemcomitans* LPS had no cytotoxic effect (data not shown). To determine whether the interactions between the two cell types modified the response to LPS stimulation, the secretion of IL-6 and IL-8 by each cell type and by the 3D co-culture model was determined by ELISA. While the stimulation of the HGF-1 cells with LPS did not significantly increase IL-6 and IL-8 secretion, the stimulation of the OBA-9 cells with LPS resulted in the secretion of higher amounts of both cytokines compared to the unstimulated cells (Figure 3). More specifically, the secretion of IL-6 and IL-8 by OBA-9 cells increased by 97% and 120%, respectively, in the presence of LPS. Interestingly, the LPS-stimulated 3D co-culture model had a synergistic response with respect to the secretion of IL-6 and IL-8 compared to that of the individual cell types (Figure 3), secreting 475 pg/mL of IL-6 and 756 pg/mL of IL-8 compared to 239 pg/mL and 496 pg/mL, respectively, by the individual cell lines. No synergistic effect was observed in the absence of LPS stimulation (Figure 3).

Prior to investigating the anti-inflammatory potential of hBD-3 and LL-37 in the 3D co-culture model, we determined their effect on the viability of LPS-stimulated gingival epithelial cells and gingival fibroblasts. None of the concentrations of hBD-3 (5, 10, 20, and 40 μM) and LL-37 (0.05, 0.1, 0.2, 0.5, 1, and 5 μM) tested had a significant effect on the viability of either cell type (Figure 4A). We then determined the effect of combinations of

concentrations of hBD-3 (10 and 20 μM) and LL-37 (0.05, 0.1, 0.2, 0.5, and 1 μM) on cell viability. None of the combinations of hBD-3 and LL-37 had a cytotoxic effect on the OBA-9 and HGF-1 cells (Figure 4B).

The LPS-stimulated 3D co-culture model was then used to investigate the anti-inflammatory properties of hBD-3 (10 and 20 μM) and LL-37 (0.1 and 0.2 μM) alone and in combination. The concentrations of 41 cytokines, chemokines, and growth factors in the cell-free culture supernatants were assayed using a multiplex ELISA assay. Only G-CSF, GRO-α, IP-10, IL-6, IL-8, and MCP-1 were detected in the culture supernatants. *A. actinomycetemcomitans* LPS significantly increased the secretion of G-CFS (36-fold), GRO-α (8-fold), IP-10 (20-fold), IL-6 (10-fold), IL-8 (20-fold), and MCP-1 (5-fold) by the 3D co-culture model compared to the unstimulated control (Figure 5). While the secretion of the cytokines was significantly reduced by 10 and 20 μM hBD-3 and by 0.1 and 0.2 μM LL-37 alone, only the secretion of GRO-α and IP-10 was significantly reduced by all the concentrations of hBD03 and LL-37 tested following the stimulation of the 3D co-culture model with LPS. hBD-3 (20 μM) and LL-37 (0.1 μM) in combination synergistically inhibited the secretion of five of the six cytokines (G-CSF, GRO-α, IP-10, IL-6, and MCP-1) by the LPS-stimulated 3D co-culture model (Figure 5). All the concentrations of hBD-3 and LL-37 tested in combination had a synergistic inhibitory effect on the secretion of G-CSF. None of the concentrations of hBD-3 and LL-37 tested in combination had a synergistic inhibitory effect on the secretion of IL-8.

Discussion

hBD-3 and LL-37 display anti-inflammatory activity in monocultures of fibroblasts, monocytes, macrophages, and periodontal ligament cells [44–47]. We investigated the anti-inflammatory activities of hBD-3 and LL-37 using a 3D co-culture model of gingival epithelial cells and fibroblasts, the two main cell types of the periodontium. We also investigated the ability of these two peptides to synergistically reduce cytokine secretion by the 3D co-culture model. We chose *A. actinomycetemcomitans* LPS to stimulate the model based on a previous study showing that *A. actinomycetemcomitans* LPS induced the highest inflammatory response of the LPS isolated from a number of periodontopathogens [48].

The 3D co-culture model is advantageous in that it takes interactions between gingival epithelial cells and fibroblasts into consideration. It has previously been shown that gingival fibroblasts stimulate the proliferation of keratinocytes, while keratinocytes induce the expression of specific fibroblast genes [31,49]. Gron et al. [50] reported that cultivating oral fibroblasts and keratinocytes together increases the secretion of keratinocyte and hepatocyte growth factors, thus modulating the proliferation and migration of the junctional epithelium. In a similar vein, our results indicated that the amounts of IL-6 and IL-8 secreted by the LPS-stimulated 3D co-culture model are significantly higher than the amounts secreted by the individual cell lines.

There has been growing interest in recent years in the synergistic antimicrobial and anti-inflammatory properties of various compounds, especially given that many diseases, including periodontitis, have a multifactorial etiology. In this regard, there may be advantages to combining different compounds to treat periodontitis. During periodontitis, periodontopathogens activate the host inflammatory response, resulting in the secretion of

pro-inflammatory mediators, which in turn modulate the destruction of tooth-supporting tissues. Compounds such as AMPs that possess both antimicrobial and anti-inflammatory properties may be potential alternatives to antibiotics in adjunctive therapies for treating periodontitis [51]. In addition to their antimicrobial properties, some AMPs can modulate the immune response and can bind directly to LPS, preventing the binding of LPS to the CD14 receptor and thus inhibiting the secretion of some pro-inflammatory cytokines. Some AMPs can also bind to LPS when it is already bound to macrophage receptors [30,52–55]. While a wide variety of human AMPs have been identified, cathelicidins and defensins are the two most thoroughly characterized families [17]. LL-37 is the only member of cathelicidin family present in humans. It possesses a broad range of immunomodulatory activities that allow it to interact with host cell membrane receptors and to inhibit the interaction between these receptors and pathogens [17,56,57]. The peptide LL-37 is expressed by by epithelial cells, monocytes, NK-cells and others cells. Defensins are found in humans, animals, and plants, and can interact with and disrupt the lipid membranes of pathogens, causing the bacteria to lyse [58,59]. Specially hBD-3 are expressed by epithelial cells, and also induced by wouding, LPS or even by inflammatory cytokines [17].

We selected hBD-3 and LL-37 to investigate their anti-inflammatory synergistic effect in the 3D co-culture model for several reasons. First, since these AMPs belong to different families, we hypothesized that they are more likely to act in synergy given that hBD-3 modulates the immune response by binding to the TLR4 receptor, that LL-37 can bind to the TLR1/2 and TLR4 receptors [34,46], and that the combination of the two peptides can inhibit the cellular signaling pathway trigged by the most important TLRs (4, 1/2) involved in LPS binding. Second, hBD-3 is the predominant defensin in the oral cavity and is produced and stored by cells in the gingival epithelium [60–62]. Lastly, many investigators have reported that hBD-3

and LL-37 possess anti-inflammatory properties [44–47], although the peptides were tested individually. Pingel et al. [63] reported that hBD-3 can significantly decrease the secretion of IL-6, IL-10, GM-CSF, and TNF-α by human myeloid dendritic cells stimulated with recombinant *Porphyromonas gingivalis* hemagglutinin B (rHagB), while Semple et al. [46] showed that hBD-3 inhibits the secretion of TNF-α and IL-6 by macrophages stimulated with *Escherichia coli* LPS. LL-37 is a potent LPS-neutralizing peptide [18,55] and strongly suppresses *E. coli* LPS- and *P. gingivalis* LPS-induced IL-6, IL-8, and CXCL 10 secretion by gingival fibroblasts [40]. In addition, Lee et al. [14] recently reported that LL-37 suppresses the pro-inflammatory activities of LPS from *Prevotella intermedia* and *Tannerella forsythia* in both monocytes and gingival fibroblasts.

Treponema denticola, *T. forsythia*, and *P. gingivalis* have been shown to either degrade or suppress the expression of hBD-3, thus perturbing the innate immune barrier of gingival epithelial cells [26,27]. Given this, combining AMPs might be an interesting strategy for increasing their anti-inflammatory and antimicrobial effects.

It is still unclear whether hBD-3 and LL-37 are pro-inflammatory or anti-inflammatory [46]. hBD-3 may behave like LL-37, which is a multifunctional modulator of the immune response and which is pro-inflammatory at high concentrations and anti-inflammatory activity at lower concentrations [64], This may explain why in the figure 5 the peptide hBD-3 at low concentrations (10 μM) was able to reduce the production of G-CSF and IL-8, although at higher concentration (20 μM) hBD-3 was not able to significantly reduce the production of those cytokines. We investigated the effect of hBD-3 and LL-37, individually and in combination, on cytokine secretion by the 3D co-culture model of gingival epithelial cells and fibroblasts stimulated with A. *Actinomycetemcomitans* LPS. This model provides a better

interpretation of the inflammatory process since it takes the synergistic effect on cytokine secretion that they may have on the two cell types into consideration.

The 3D co-culture model secreted higher levels of MCP-11, GRO-α, IL-6, and IL-8 and, to a lesser extent, IP-10 and G-CSF in response to *A. actinomycetemcomitans* LPS. All of these molecules may contribute in different ways to the progression of periodontitis. As reported by Sager et al. [65], GRO-α induces an intense inflammatory response when injected into mice, contributing to the degradation of the extracellular matrix and promoting leukocyte infiltration. Kurtis et al. [9] reported that the concentration of MCP-1 in the GCF from diseased sites is significantly higher than in the GCF from healthy sites. IL-6 and IL-8 are important inflammatory mediators secreted by macrophages, fibroblasts, and epithelial cells and are found in high concentrations in inflamed gingival and periodontal tissues [66–68]. Almasri et al. [69] reported that gingival fibroblasts stimulated with *P. gingivalis* LPS secrete more GRO-α, IL-6, IL-8, and MCP-1 than unstimulated controls, which is in agreement with our results.

Ours results showed that 10 μM hBD-3 and 0.2 μM LL-37 alone significantly reduce the secretion of G-CSF, GRO-α, IL-6, IL-8, IP-10, and MCP-1 by the 3D co-culture model in response to LPS. In addition to the anti-inflammatory property of each peptide alone, the combination of 20 μM hBD-3 and 0.1 μM LL-37 synergistically reduced the secretion of GM-SCF, GRO-α, IL-6, IP-10, and MCP-1 in response to LPS. However, the combination of hBD-3 and LL-37 only had an additive effect on reducing the secretion of IL-8 at all the concentrations tested compared to the 3D co-culture model.

To the best of our knowledge, nobody has reported that a combination of AMPs has a synergistic anti-inflammatory effect. Although, Semple et al. [46] showed that the association

of hBD-3 and 8-bromoadenosine-cAMP (8Br-cAMP) a membrane permeable cAMP analogue reduces the secretion of TNF-α by mouse macrophages (RAW 264.7) more than hBD-3 or 8Br-cAMP alone. Semple et al. [46] concluded that hBD-3 acts through toll-like receptor 4 (TLR4) while LL-37 acts through both TLR4 and TLR1/2 [70]. The combination of the two peptides used in this manuscript (hBD-3 and LL-37) may thus be more effective in reducing pro-inflammatory cytokine secretion because they act through different signaling pathways.

We used a periodontal relevant 3D co-culture model composed of gingival epithelial cells and fibroblasts to show that the AMPs hBD-3 and LL-37 both reduce the secretion of inflammatory cytokines and thus display anti-inflammatory activity. Moreover, combining the two peptides produced a synergistic effect.

Conclusion

The 3D co-culture model used in the present study takes the interactions that may occur between different cell types (epithelial cells and fibroblasts) into consideration and thus mimics the in vivo condition more accurately than does studying each cell type separately. The 3D co-culture model produced a synergistic increase in the secretion of IL-6 and IL-8 following *A. actinomycetemcomitans* LPS stimulation compared to the individual cell lines. In addition, while hBD-3 and LL-37 both displayed anti-inflammatory activity when applied individually to the model, they acted in synergy when applied together. This suggests that the combination of the two AMPs could be a valuable strategy for replacing antibiotics in adjunctive therapies for the treatment of periodontitis. Further studies are required to investigate the effect of hBD-3 and LL-37 in vivo.

Conflict of Interests

The authors declare that they have no conflicts of interests with respect to the work reported in this article.

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Figures

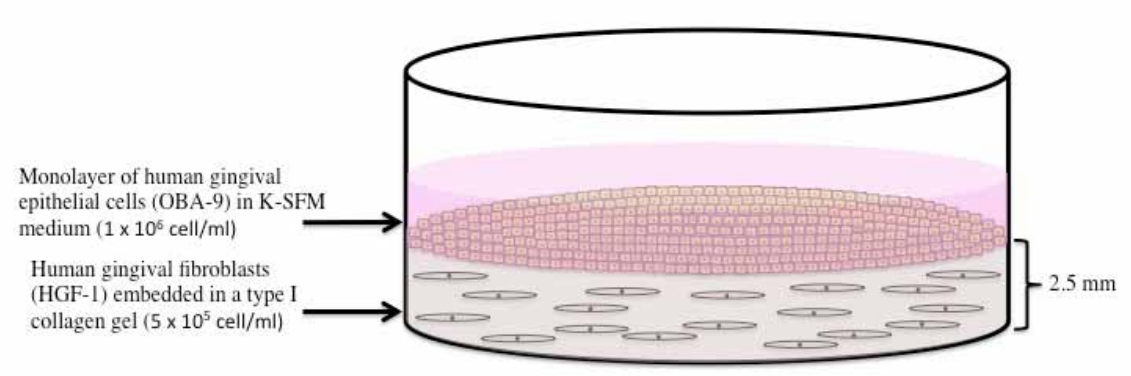

Figure 1: Schematic representation of the 3D co-culture model. The model is composed of gingival fibroblasts (HGF-1) embedded in a collagen matrix overlaid with gingival epithelial cells (OBA-9) and is a modification of the model described by Gursoy et al. [22].

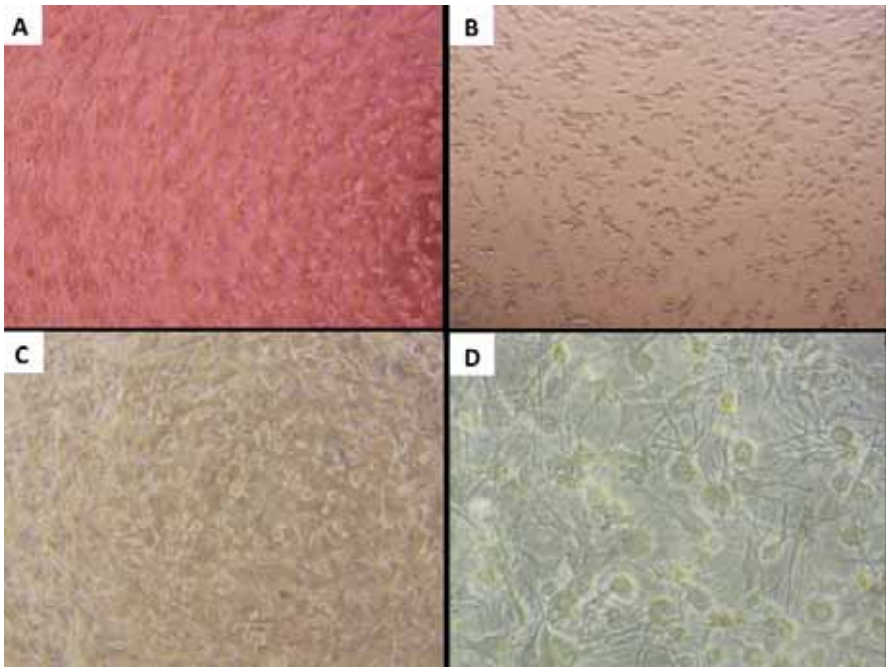

Figure 2: Light microscopic images of the individual cell lines and the 3D co-culture model. A: Collagen-fibroblast gel in DMEM; B: Gingival epithelial cells seeded on the collagen gel; C and D: 3D co-culture model composed of gingival fibroblasts embedded in a collagen matrix overlaid with gingival epithelial cells. The epithelial cells, fibroblasts, and 3D co-culture model were stimulated with *A. actinomycetemcomitans* LPS (1 µg/mL) for 24 h at 37C in a 5% CO_2 atmosphere. Panels A, B, and C (4x magnification). Panel D (10x magnification).

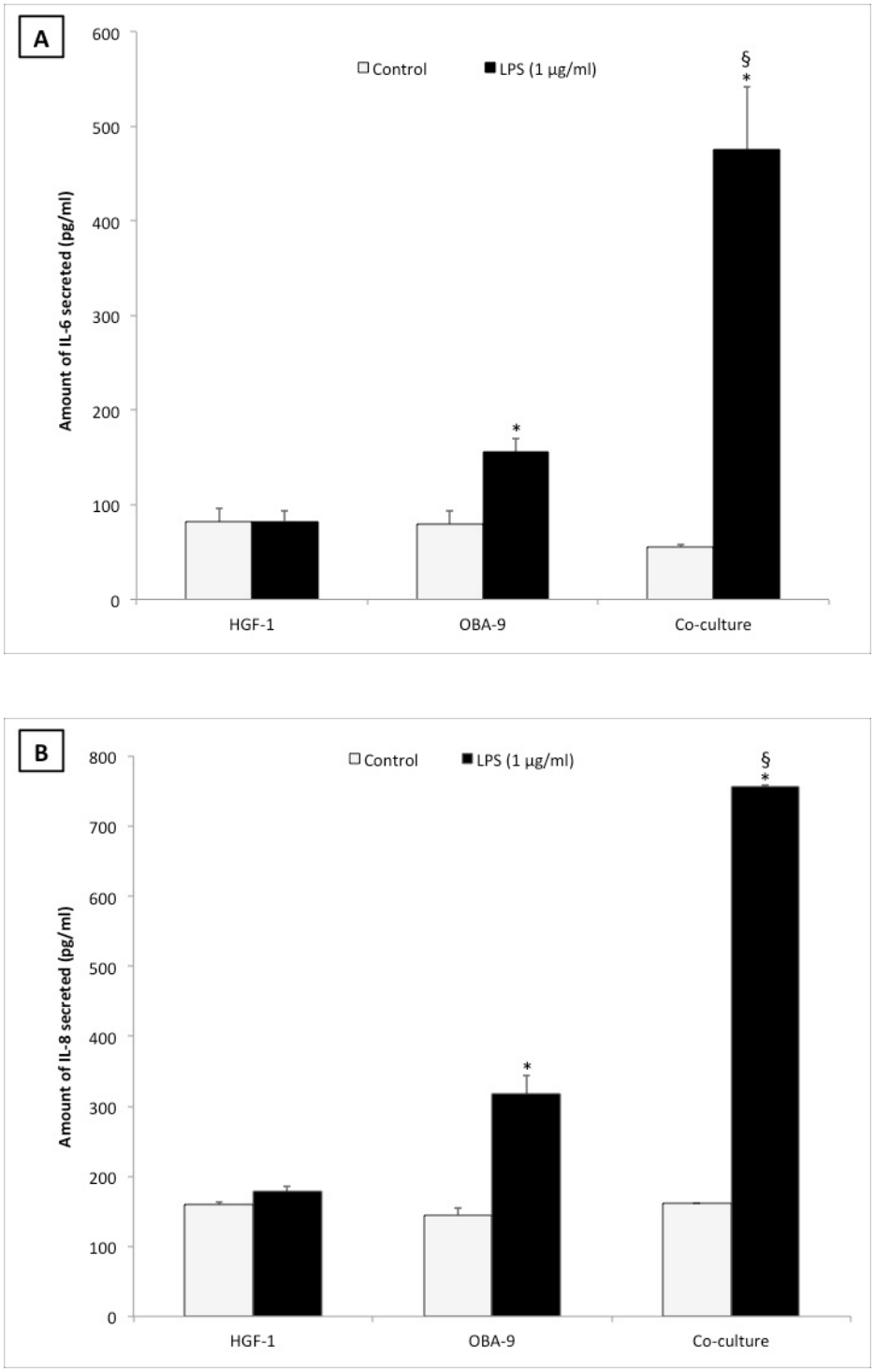

Figure 3: Amount of IL-6 (A) and IL-8 (B) secreted by gingival fibroblasts (HGF-1), gingival epithelial cells (OBA-9), and the 3D co-culture model in the absence and presence of *A. actinomycetemcomitans* LPS (1 µg/mL). Results are expressed as means ± standard deviation of triplicate assays from two independent experiments. ∗, $p < 0.05$: significantly different from the control cells without LPS stimulation for the individual cell lines and the 3D co-culture model; §, $p < 0.05$: synergistic effect of the cells in the 3D co-culture model compared to the individual cell lines.

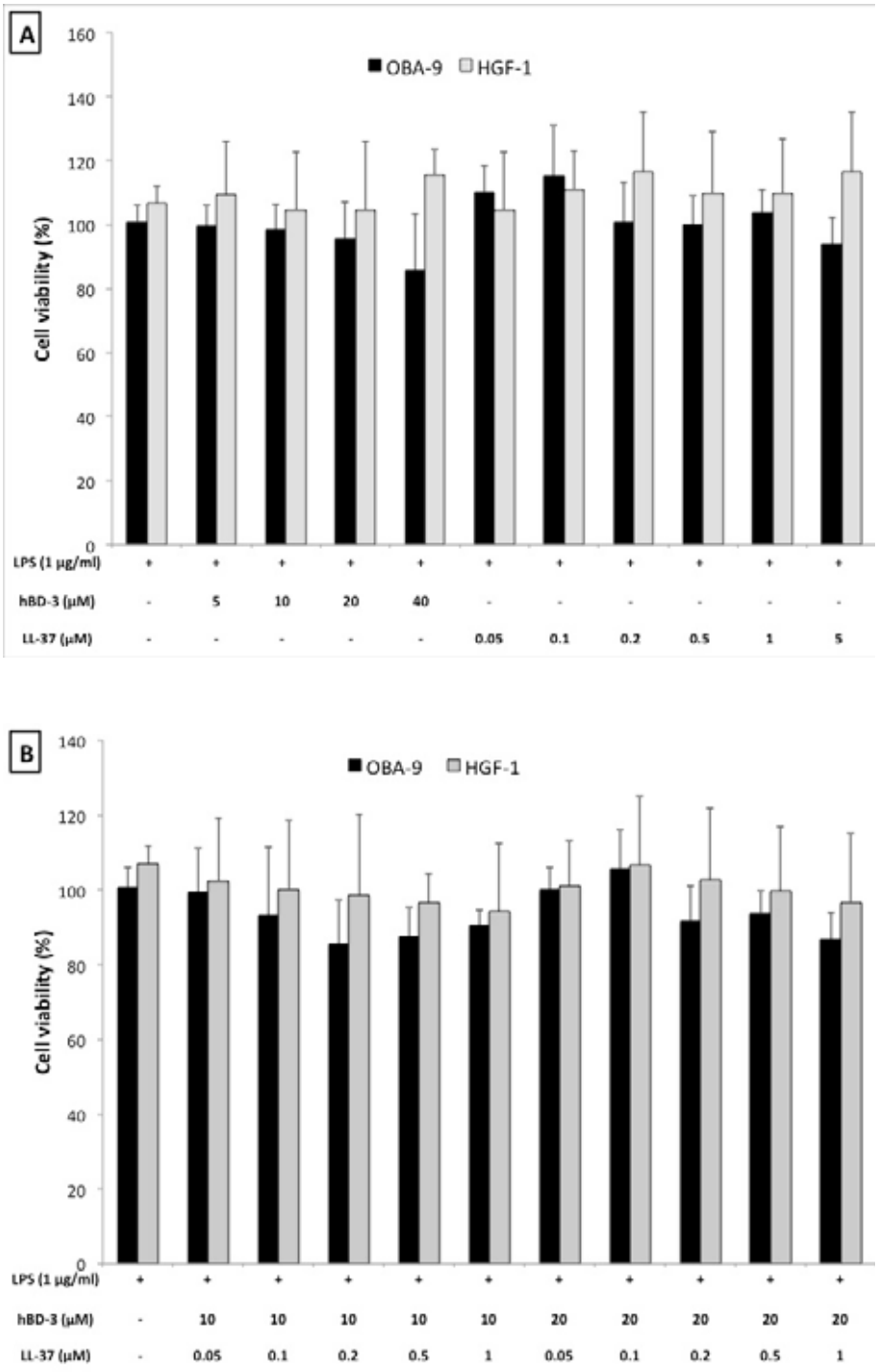

Figure 4: Effect of hBD-3 and LL-37 alone (A) and in combination (B) on the viability of LPS-stimulated gingival fibroblasts (HGF-1) and gingival epithelial cells (OBA-9). Untreated cells were assigned a value of 100%. All the other stimulations were compared to the control. Results are expressed as means ± standard deviation of duplicate assays from two independent experiments. No statistical significance was observed using ANOVA.

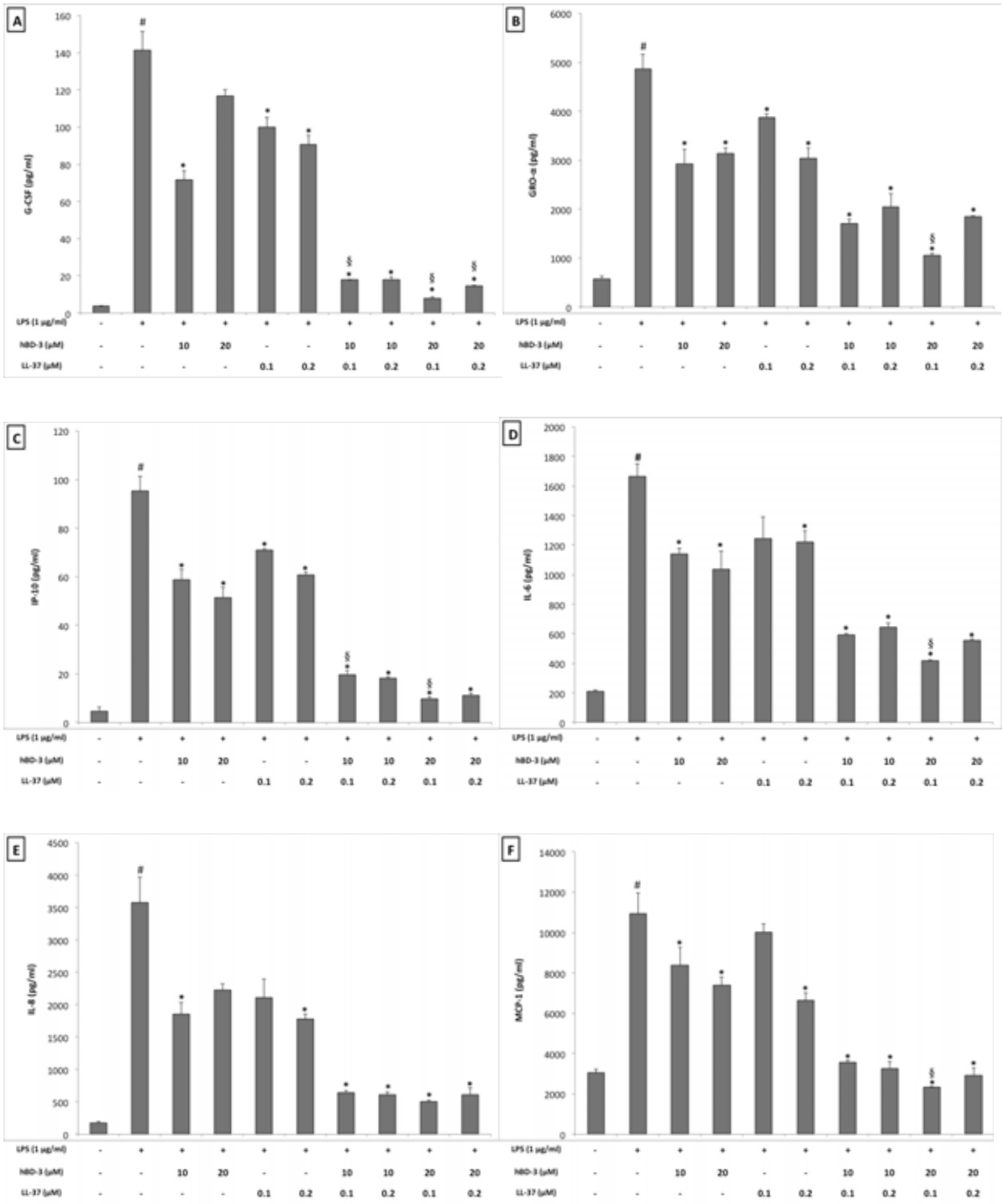

Figure 5: Effect of hBD-3 and LL-37 alone and in combination on the secretion of G-CFS (A), GRO-α (B), IP-10 (C), IL-6 (D), IL-8 (E), and MCP-1 (F) by the LPS-stimulated 3D co-culture model. Results are expressed as means ± standard deviation of triplicate assays from two independent experiments. #, significantly higher than the unstimulated (LPS) negative control ($p < 0.01$); *, significantly lower than the untreated (hBD-3, LL-37) positive control ($p < 0.05$); §, synergistic effect of the peptides; significantly lower than the sum of the inhibitory values of each peptide alone ($p < 0.05$) compared to the two peptides in combination.

Capítulo 3

Artigo a ser submetido
Journal of Periodontology

Green tea polyphenol epigallocatechin-3-gallate and cranberry proanthocyanidins act in synergy with cathelicidin (LL-37) to reduce the LPS-induced inflammatory response in a 3-dimensional co-culture model of gingival epithelial cells and fibroblasts

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Short title: Synergistic anti-inflammatory effect of LL-37 with EGCG and AC-PACs

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ABSTRACT

The antimicrobial peptide cathelicidin (LL-37) possesses anti-inflammatory properties that may contribute to attenuate the inflammatory process associated with chronic periodontitis. Moreover, plant polyphenols, including those from green tea and cranberry, have also been reported to reduce inflammatory cytokine secretion by host cells. In the present study, we used a three-dimensional (3D) co-culture model of gingival epithelial cells and fibroblasts stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) to investigate the ability of A-type cranberry proanthocyanidins (AC-PACs) and epigallocatechin-3-gallate (EGCG) to act in synergy with the antimicrobial peptide LL-37 to regulate the secretion of inflammatory mediators. The LPS-stimulated co-culture model was treated with non-cytotoxic concentrations of AC-PACs (25 and 50 μg/ml), EGCG (1 and 5 μg/ml) and LL-37 (0.1 and 0.2 μM), individually, as well as with the combinations AC-PACs + LL-37 and EGCG + LL-37. A Multiplex ELISA assay was used to quantify the secretion of 54 different host factors, including chemokines, cytokines, growth factors, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). While LL-37, AC-PACs, and EGCG, individually or in association, had no effect on the regulation of MMPs and TIMPs secretion, they can inhibit the secretion of specific cytokines. On the one hand, AC-PACs and LL-37 acted in synergy to reduce the secretion of CXC-chemokine ligand 1 (GRO-α), granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), while they only had an additive effect to reduce the secretion of interleukin-8 (IL-8), interferon-γ inducible protein 10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1) in response to LPS stimulation. On the other hand, the combination EGCG + LL-37 showed a synergistic effect to reduce the secretion of GRO-α, G-CSF, IL-6, IL-8 and IP-10 while only an additive effect on MCP-1 secretion. In conclusion, the association of natural polyphenols

with the antimicrobial peptide LL-37 resulted in synergistic interactions in regard to the secretion of cytokines by an LPS-stimulated 3D co-culture model. Consequently, such combinations may offer perspective as potential adjunctive therapy for inflammatory periodontitis.

Keywords: catechin, cathelicidin, cranberry, green tea, epithelial cell, fibroblast, periodontal disease, proanthocyanidin,

Introduction

Periodontitis is a multifactorial chronic inflammatory disease with a polymicrobial origin that causes destruction of the tooth-supporting tissues, including the periodontal ligament and alveolar bone [1]. It is initiated by a limited number of Gram-negative bacteria that colonize the subgingival area and activate the host immune response [1]. More specifically, the lipopolysaccharide (LPS) of *Aggregatibacter actinomycetemcomitans*, the key etiologic agent of aggressive periodontitis, is recognized as an important virulence factor [2, 3]. This endotoxin found in the bacterial outer membrane can promote adhesion to and invasion of oral mucosal cells, and consequently activation of the host immune response resulting in secretion of high amounts of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) which modulate periodontal tissues destruction[4-6].

Gingival epithelial cells are the primary physical barrier to tissue invasion by periodontopathogens, while gingival fibroblasts are the predominant cells of the periodontal connective tissue and play important roles in periodontal tissue repair and inflammatory processes induced by periodontopathogens [7]. Both cell types are able to secrete antimicrobial peptides, which are small cationic molecules of the innate immunity, possessing a broad activity spectrum against pathogens [8, 9]. Antimicrobial peptides play a significant role in the host innate immune defense to prevent infections [10-14]. Cathelicidins are an important class of mammalian antimicrobial peptides. The peptide hCAP18 is the only cathelicidin found in humans; its C-terminal end is proteolytically cleaved to generate an active 37-amino acid peptide beginning with two leucine residues, from which the name LL-37 is derived [15, 16]. LL-37, which is expressed by several cell types, including monocytes, neutrophils and epithelial cells, is known to possess pleiotropic effects [12, 17-19]. More specifically, LL-37 reduces *P.*

gingivalis killed cells and LPS induced IL-6, IL-8, nitric oxide (NO) and tumor necrose factor (TNF- α) production on human gingival fibroblasts [20] and on bone marrow-derived macrophages (BMDM) [21]. Was also reported that LL-37 is able to reduce pro-inflammatory cytokine, such as IL-6 and IL-8, by oral cells through inhibit the ligand recognition of TLRs and also through the directing binding of LL-37 to LPS, so inhibit interaction between LPS and CD14 receptor [22, 23]. LL-37 has been detected in gingival crevicular fluid and saliva [19, 24, 25]. The levels of LL-37 in gingival crevicular fluid have been reported to be lower in diseased periodontal sites when compared to healthy sites [26, 27].

Given the multifactorial etiology of periodontitis, a combination of drugs may provide a number of benefits resulting in a better therapeutic outcome. This is likely associated to synergistic effects occurring between compounds and allowing to get the same or better therapeutic treatment results with lower dosage of drugs and cytotoxicity [28]. In this regard, phytochemicals are considered as promising bioactive molecules being able to exert their properties on both the infective agent and the host. Cranberry (*Vaccinium macrocarpon*) polyphenols have received attention as potential therapeutic agents for the prevention of many human diseases, including cancers, cardiovascular diseases and infectious diseases [29-32]. Proanthocyanidins from cranberry are characterized by the presence of unusual structures with A-type linkages and a second ether linkage between an A-ring of the lower unit and the C-2 ring of the upper unit (O7 C2) [33]. In recent years, a number of studies have brought evidence regarding beneficial properties of cranberry proanthocyanidins for periodontal disease, through their capacity to inhibit biofilm formation, tissue-destructive enzymes and secretion of inflammatory cytokines by immune and mucosal cells[34-37]. Green tea (*Camellia sinensis*) polyphenols are mostly catechins; the most predominant being epigallocatechin-3-gallate (EGCG) for which several beneficial pharmacological properties have been identified

[38]. In regard to periodontal disease, EGCG has been shown to inhibit the growth of important periodontopathogens [39] to interfere with osteoclast formation [40], and to reduce the secretion of pro-inflammatory cytokines by gingival fibroblasts[41], epithelial cells [42] and endothelial cells [43]. Interestingly, Hirasawa et al. (2002)[44] reported that local delivery of green tea catechins inside the periodontal pockets could enhance the success of conventional periodontal treatments.

To the best of our knowledge, there are no data in the literature on potential synergistic effects between polyphenols and the antimicrobial peptide LL-37 in regard to anti-inflammatory properties. Consequently, the aim of this study was to evaluate the potential synergistic interactions of AC-PACs or EGCG when used in combination with LL-37 on inhibition of pro-inflammatory mediator, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) secretion by a 3D co-culture model of epithelial cells and fibroblasts stimulated with *A. actinomycetemcomitans* LPS.

Material and methods

LL-37, A-type cranberry proanthocyanidins (AC-PACs), epigallocatechin-3-gallate (EGCG), and LPS

The synthetic LL-37 peptide (H-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-OH) were purchased from BIOMATIK Company (Cambridge, ON, Canada) and dissolved in sterile UltraPure™ DNase/RNase-free distilled water (Life Technologies Inc., Burlington, ON, Canada) at a concentration of 1 mM and stored at -20°C until used. AC-PACs were isolated from cranberry juice concentrate according to the protocol described in a previous study [45]. AC-PACs were dissolved in 70% ethanol at a final concentration of 20 mg/ml and stored at 4°C until used. **EGCG** was purchased from Sigma-Aldrich Canada. (Oakville, ON, Canada). EGCG was dissolved in sterile warm distilled water at a concentration of 2 mg/ml, sterilized by filtration using a **0.2-µm-pore membrane**. *A. actinomycetemcomitans* (ATCC 29522) LPS, isolated using the protocol described by Darveau & Hancock (1983) [46] , was prepared at a concentration of 1 mg/ml in sterile distilled water, and stored at -20°C until used.

Cultivation of gingival epithelial cells and fibroblasts

The immortalized human gingival epithelial cell line OBA-9 [47], which was kindly provided by M. Mayer (Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil), was cultured in keratinocyte serum-free medium (K-SFM; Life Technologies Inc.) containing insulin, epidermal growth factor, fibroblast growth factor and penicillin G-streptomycin (100 µg/ml). The primary human gingival fibroblast cell line HGF-1 (ATCC® CRL-2014) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA), 10% heat-inactivated fetal bovine serum (FBS), and 100 µg/ml of penicillin G-streptomycin. Both cell lines were incubated at 37°C in a 5% CO_2 atmosphere until they reached confluence.

Preparation of the three-dimensional (3D) co-culture model

A 3D co-culture model composed of gingival fibroblasts embedded in a collagen matrix and overlaid with gingival epithelial cells was prepared according to the protocol described by Gursoy et al. (2012) [48], with slight modifications. A commercial bovine type I collagen solution (95-98%; PureCol, Advanced BioMatrix, Tucson, AZ, USA) was mixed with DMEM (10X) (Sigma-Aldrich Canada) on ice to obtain a final collagen concentration of 76-78% adjusted at pH 7. Confluent HGF-1 cells were detached by gentle trypsinization (0.05% trypsin-EDTA; Gibco-BRL, Grand Island, NY, USA). The trypsin was then inactivated by adding DMEM + 10% FBS. The cells were harvested by centrifugation (500 x *g* for 5 min) and were suspended at a density of 5 x 10^5 cells/ml in the collagen solution described above. The collagen cell suspension was placed into wells of 6-well tissue culture plates (2 ml/well). The collagen gel (approximately 2.5-mm-thick) was allowed to solidify for 2 h at 37°C under aerobic conditions, and the plates were then incubated for a further 10 h at 37°C in a 5% CO_2 atmosphere. The OBA-9 cells were detached by gentle trypsinization (5 min) (TrypLE™ Express; Life Technologies Inc.) at 37°C. The trypsin was then inactivated by adding 0.3 mg/ml of trypsin inhibitor, and the cells were harvested by centrifugation (500 x *g* for 5 min) and suspended in fresh K-SFM medium. Aliquots (2 ml) of OBA-9 cells were seeded on top of the collagen-fibroblast gels at a density of 1 x 10^6 cell/ml. The 3D co-culture model was incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion prior to stimulation.

Stimulation of the 3D co-culture model

The 3D co-culture model was pre-treated during 2 h with AC-PACs (25, 50 µg/ml), EGCG (1 and 5 µg/ml) or LL-37 (0.1 and 0.2 µM) or with the combination AC-PACs + LL-37 and EGCG + LL-37, prior to stimulate with *A. actinomycetemcomitans* LPS (1 µg/ml) for 24 h at 37°C in a 5% CO_2 atmosphere. Co-cultures not pre-treated with AC-PACs, EGCG and LL-37 as well as not stimulated with LPS were used as controls. The supernatants were collected, subjected to centrifugation (1000 x *g* for 5 min at 4°C), and then stored at - 20°C until used.

Determination of cell viability

The effect of AC-PACs, EGCG, LL-37, and *A. actinomycetemcomitans* LPS, individually and in association, on the viability of gingival epithelial cells (OBA-9) and gingival fibroblasts (HGF-1) was evaluated. Briefly, human cells (1 x 10^4 cells/well) were seeded in the wells of a 96-well microplate (0.1 ml/well) and incubated for 4 h at 37°C in a 5% CO_2 atmosphere to allow cell adhesion. The culture medium was then aspirated, and the cells were pre-treated for 2 h with AC-PACs (6.25, 12.5, 25, 50, 100 µg/ml), EGCG (1, 5, 10, 25, 50 µg/ml) or LL-37 (0.05, 0.1, 0.2, 0.5, 1, 5 µM), individually, or with the combination AC-PACs (6.25, 12.5, 25, 50, 100 µg/ml) + LL-37 (0.1, 0.2 µM), and EGCG (1, 5, 10, 25, 50 µg/ml) + LL-37 (0.1, 0.2 µM) prior to stimulate with *A. actinomycetemcomitans* LPS (1 µg/ml). The cells were incubated for an additional 24 h at 37°C in a 5% CO_2 atmosphere. A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany) using 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as the substrate was performed according to the manufacturer's protocol. Untreated control cells were assigned a value of 100%, and all the other conditions were compared to the control. Results were expressed as means ± standard deviations of duplicate assays from two independent experiments.

Determination of cytokines, MMPs and TIMPs secretion using multiplex ELISA assays

Samples of the 3D co-culture model subjected to the various treatments were sent to Eve Technologies (Calgary, AB, Canada; http://www.evetechnologies.com) for multiplex ELISA analyses. Eve Technologies uses the Bio-Plex Suspension Array System to quantify 41 different cytokines, chemokines, and growth factors (Human 41-Plex Discovery Assay): epidermal growth factor (EGF), C-C motif chemokine 11 (Eotaxin-1), basic fibroblast growth factor (FGF-2), FMS-like tyrosine kinase 3 ligand (Flt3l), chemokine (C-X3-C motif) ligand 1 (Fractalkine), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), CXC-chemokine ligand 1 (GRO-α), interferon alpha 2 (IFN-α2), interferon gamma (IFNγ), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-12B (IL-12B), interleukin-12 (p70) [IL-12 (p70)], interleukin-13 (IL-13), interleukin-15 (IL-15), interleukin 17A (IL-17A), interferon-γ inducible protein 10 (IP-10), monocyte chemo-attractant protein 1 (MCP-1), monocyte-specific chemokine 3 (MCP-3), C-C motif chemokine 22 (MDC), macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), platelet-derived growth factor AA (PDGF-AA), platelet-derived growth factor AB/BB (PDGF-AB/BB), regulated on activation, normal T cell expressed and secreted (RANTES), soluble CD40 ligand (sCD40L), transforming growth factor alpha (TGF-α), tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), vascular endothelial growth factor A (VEGF-A), as well as nine MMPs (-1, -2, -3, -7, -8, -9, -10, -12 and -13; Human MMP 9-Plex Discovery Assay) and four TIMPs (-1, -2, -3 and -4; Human TIMP 4-Plex Discovery Assay).

Data analysis

To determine the synergistic anti-inflammatory effect of the peptide LL-37 in association with AC-PACs and EGCG in the 3D co-culture model stimulated with *A. actinomycetemcomitans* LPS, the sums of inhibition values of i) AC-PACs + LL-37, and ii) EGCG + LL-37 were compared with the values obtained when compounds were used in association. Experiments were carried out a minimum of three times to ensure reproducibility, and means $\pm$ SD from a representative experiment is presented. Differences between the means were analyzed for statistical significance using a one-way ANOVA. Statistical significance was set at $p < 0.05$.

Results

Prior to investigate the capacity of the natural compounds AC-PACs and EGCG, individually and in association with LL-37, to regulate the secretion of inflammatory mediators, MMPs, and TIMPs, we determined their effect on the viability of gingival epithelial cells (OBA-9) and gingival fibroblasts (HGF-1). Our results showed that the treatment (24 h) of both cell lines with AC-PACs, EGCG and LL-37, at all the concentrations tested, individually and in association, in the presence of 1μg/ml of *A. actinomycetemcomitans* LPS had no cytotoxic effect as evaluated by an MTT test (data not shown). More specifically, cell viability was higher than 90% for each stimulation when compared with untreated control cells. This supports that the capacity of both natural compounds, individually and in association with LL-37, to regulate the secretion of inflammatory mediators will not be related to cell toxicity.

The LPS-stimulated 3D co-culture model was then used to investigate the capacity of AC-PACs and EGCG, individually and in association with the peptide LL-37, to modulate the secretion of cytokines, chemokines, growth factors, MMPs and TIMPs determined with a multiplex ELISA analysis. Among the 41 different cytokines, chemokines, and growth factors determined, only G-CSF, GRO-α, IP-10, IL-6, IL-8, and MCP-1 were detected in the culture supernatants of the co-culture model. *A. actinomycetemcomitans* LPS significantly increased the secretion of G-CFS (36-fold), GRO-α (8-fold), IP-10 (20-fold), IL-6 (10-fold), IL-8 (20-fold), and MCP-1 (5-fold) by the 3D co-culture model compared to the unstimulated control (Figure 1 and 2).

We then investigated in the 3D co-culture model the ability of AC-PACs (25 and 50 μg/ml) and EGCG (1 and 5 μg/ml) individually or in association with the peptide LL-37 (0.1 and 0.2 μM) to reduce the secretion of the above host factors. Our results showed no regulation by these compounds, individually or in association, on the secretion of MMPs (-1, -2, -3, -7, -8, -9, 10, -12 and -13) and TIMPs (-1, -2, -3 and -4) (data not shown). When used individually, AC-PACs (25 and 50 μg/ml respectively) were able to significantly reduce the secretion of G-CFS, GRO-α around 34% and 45% respectively, IL-8, IP-10 around 67% and 80% respectively and AC-PAC at both concentration studied was also able to reduce 70% the secretion of MCP-1, while they did not affect the secretion of IL-6 (Figure 1). Moreover, EGCG alone, although only at the concentration of 5 μg/ml, was able to significantly reduce the secretion of GRO-α, IP-10, IL-8 and MCP-1 around 15%, 17%, 29% and 60% respectively, but did not have effect on the secretion of G-CSF and IL-6 (Figure 2).

The anti-inflammatory activity of the compounds with the peptide LL-37 was then evaluated. The combination of AC-PACs at 25 μg/ml and LL-37 at 0.1 μM synergistically inhibited the secretion of G-CSF, GRO-α and IL-6 around 90%, 67% and 60% respectively by the LPS-stimulated 3D co-culture model (Figure 1). None of the concentrations of AC-PACs and LL-37 tested in association had a synergistic inhibitory effect on the secretion of IP-10, IL-8 and MCP-1, since AC-PACs and LL-37 individually were able to significantly reduce the secretion of those cytokines, although the association of AC-PACs at 25 μg/ml and LL-37 at 0.1 μM showed a significantly additive effect to reduce the secretion of IL-8, IP-10 and MPC-1 around 83%, 89% and 85% respectively. On the other hand, the association of EGCG and LL-37 synergistically inhibited the secretion of G-CSF, GRO-α, IP-10, IL-6 and IL-8, more specifically EGCG at 5 μg/ml and LL-37 at 0.1 μM in association were able to synergistically reduce the secretion of G-CSF, GRO-α, IP-10 and IL-6 around 80%, 50%, 73% and 55%

respectively. None of the concentration of EGCG and LL-37 tested in association had a synergistic inhibitory effect on the secretion of MCP-, although EGCG at 5 μg/ml and LL-37 at 0.1 μM showed an additive effect to reduce the secretion of MCP-1 around 77%.

Discussion

Periodontitis is a complex inflammatory disease with a multifactorial etiology. First, the overgrowth of specific bacterial species in subgingival sites initiates a host immune response. Then, the continuous stimulation of the immune system by pathogenic bacteria and their toxic products modulates a number of host-mediated destructive processes resulting from the accumulation of inflammatory mediators and MMPs. Consequently, drugs or natural compounds with both antimicrobial and anti-inflammatory properties are likely to provide better outcomes when used in periodontal therapy. In this regard, AC-PACs from cranberry and EGCG, which are predominant polyphenols from cranberry and green tea, respectively, represent interesting natural compounds. On the one hand, AC-PACs has been reported to inhibit adherence, biofilm formation, and protease activity of *Porphyromonas gingivalis* [49], as well as to reduce cytokines and MMPs secretion by mucosal and immune cells [45, 49-51]. On the other hand, EGCG possesses both antibacterial [39] and anti-inflammatory properties [52, 53], in addition to induce b-defensin secretion by gingival epithelial cells [54]. In this study, AC-PACs and EGCG were combined with the antimicrobial peptide LL-37 and then tested in a 3D co-culture model of gingival epithelial cells and fibroblasts stimulated with *A. actinomycetemcomitans* LPS for their capacity to synergistically inhibit the secretion of pro-inflammatory mediators, MMPs and TIMPs. To the best of our knowledge there are no data in the literature regarding the impact of an association between polyphenolic compounds and a human antimicrobial peptide.

To better investigate the anti-inflammatory activity of AC-PACs, EGCG and LL-37 we used a 3D co-culture model of gingival epithelial cells and fibroblasts cells, two predominant cell types of the periodontium. The LPS of *A. actinomycetemcomitans* was selected to induce an inflammatory response based on a previous study in our laboratory that showed that this LPS

induces the secretion of the highest amount of pro-inflammatory cytokines when compared with LPS from other periodontopathogens [34]. Stimulation of the 3D co-culture model with *A. actinomycetemcomitans* LPS induced the secretion of high amounts of MCP-1, GRO-α, IL-6 and IL-8 and to a lesser extent of IP-10 and G-CSF when were compared with un-stimulated co-culture model. *A. actinomycetemcomitans* LPS was also able to induce the secretion of MMPs and TIMPs by the 3D co-culture model, although none of the concentration of the compounds AC-PACs and EGCG individually or in association with LL-37 were able to decrease the secretion of MMPS and TIMPs (data not shown). As demonstrated previously GRO-α, MCP-1, IL-6, IL-8 are important inflammatory mediators secreted by different cell types, such as epithelial cells and fibroblasts, are detected in highest amounts in inflammation area, such as gingival crevicular fluid of patients with periodontitis when compared with health sites [55, 56]. More specifically, IL-6 and IL-8 are able to enhance of bone resorption, stimulate the attraction and activation of neutrophils and activate B-cells, that results in a non-specific antibody production [57]. On the one hand, MCP-1 is a monocyte chemotaxis and GRO-α is chemotratic for neutrophils [58, 59]. Strategies that could decrease the secretion of the inflammatory mediators may thus be beneficial for periodontal tissues [55, 60, 61].

When used individually, AC-PACs, EGCG and LL-37 were able to decrease the secretion of cytokines by the LPS-stimulated 3D co-culture model. Our results showed that AC-PACs and LL-37 were able to significantly reduce the secretion of G-SCF, GRO-α, IL-8, IP-10 and MCP-1, while only LL-37 was able to reduce the secretion of IL-6. On the other hand EGCG was able to reduce the secretion of GRO-α, IL-8, IP-10 and MCP-1 but did not affect the secretion of G-CSF and IL-6. The above observations are in agreement with previous studies that used human cells in monoculture to demonstrate anti-inflammatory activity. Indeed, it has been reported that cranberry proanthocyanidins reduce the secretion of IL-6, IL-8 and

prostaglandin E_2 (PGE_2) by fibroblasts and macrophages stimulated with *A. actinomycetemcomitans* LPS [45, 50], EGCG was reported to reduce the gene expression of IL-1β, IL-6, TNF-α, osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and RANKL/OPG by human periodontal ligament fibroblasts stimulated with *Porphyromonas gingivalis* LPS [52]. LL-37 was reported to reduce the secretion of IL-6 and IL-8 by *P. gingivalis* stimulated human gingival fibroblasts [20, 22].

In addition to the anti-inflammatory activity of AC-PACs, EGCG, and LL-37 when taken individually, we found a synergistic effect of AC-PACs in association with LL-37 in regard to reduction of G-SCF, GRO-α and IL-6 secretion by the LPS-stimulated 3D co-culture model. Moreover, the association of AC-PACs with LL-37 showed only additive effect for the reduction of IP-10, IL-8 and MCP-1 secretion. The association of EGCG with LL-37 also synergistically inhibited the secretion G-CSF, GRO-α, IL-6, IL-8 and IP-10 as compared with each compound individually.

Synergistic interactions involving AC-PACs have been previously reported. More specifically, AC-PACs has been shown to act in synergism with licochalcone A, a polyphenol belonging to the chalcone class, to reduce the secretion of IL-1β, TNF-α, IL-6 and IL-8 by macrophages cells stimulated with *A. actinomycetemcomitans* LPS [49].

The anti-inflammatory synergy observed between polyphenols and LL-37 may be related to their different mode of action. On the one hand, polyphenols such as AC-PACs and EGCG can inhibit the secretion of pro-inflammatory cytokines through the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway [62]. On the other hand, the antimicrobial peptide LL-37 is able to bind to TLR1/2 and TLR4 cell surface receptors and consequently to prevent the LPS binding and cell activation [22].

The antimicrobial peptide LL-37 can be proteolytic inactivated by *P. gingivalis* and *Tannerella forsythia* [8, 26, 63, 64]. The association of this peptide with either AC-PACs or EGCG could be an interesting strategy to protect LL-37 from proteolytic degradation. Indeed, was reported that AC-PACs at 100 µg/ml was able to significantly reduce the secretion of MMP-1, -3, -7, -8, -9, and -13 by monocyte derived macrophages stimulated with *A. actinomycetemcomitans* LPS [51]. Moreover EGCG is able to reduce *P. gingivalis* LPS enhanced secretion of MMP-1 by human gingival fibroblasts, and to inhibit the activity of protein tyrosine phosphatase (PTPase) from *Prevotella intermedia* [65, 66].

LL-37 has been detected in gingival crevicular fluid and saliva [19, 24, 25]. The levels of LL-37 in gingival crevicular fluid have been reported to be lower in diseased periodontal sites when compared to healthy sites [27].

Conclusion

Using a 3D co-culture model made of gingival epithelial cells and fibroblasts, our study showed that AC-PACs, EGCG and the antimicrobial peptide LL-37 individually exert an anti-inflammatory activity by decreasing the secretion of inflammatory mediators. In addition, the association of either polyphenolic compound (AC-PACs and EGCG) with LL-37 resulted in synergistic interactions in regard to inflammatory cytokine secretion. These observations suggest that the association of natural compounds, such as AC-PACs and EGCG with the antimicrobial peptide LL-37 could be a valuable strategy to use as a novel adjunctive therapy to periodontitis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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Figures

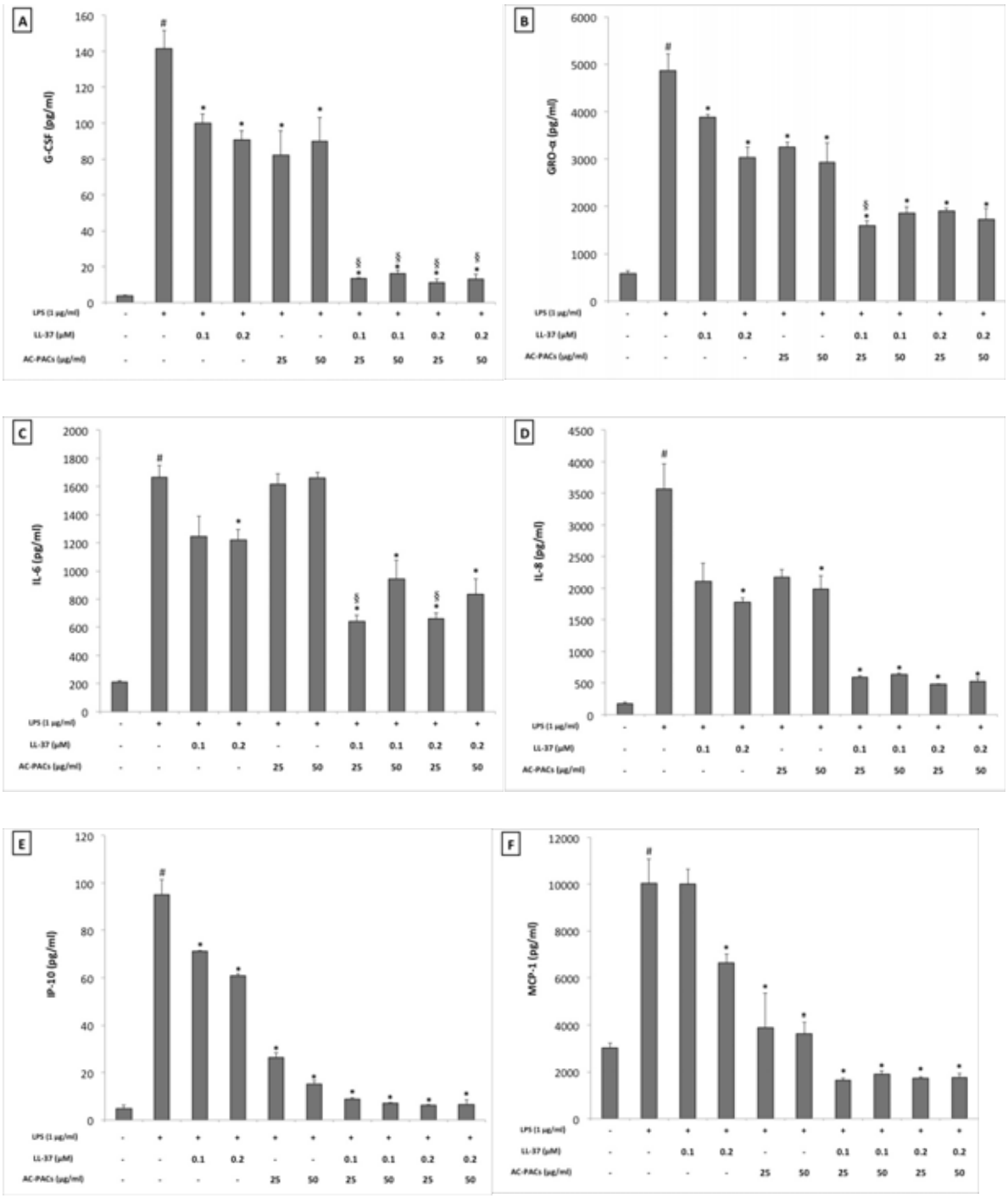

Figure 1: Effect of AC-PACs and LL-37, individually and in association, on the secretion of G-CFS (A), GRO-α (B), IL-6 (C), IL-8 (D), IP-10 (E) and MCP-1 (F) by the LPS-stimulated 3D co-culture model. Results are expressed as means ± standard deviations of triplicate assays from two independent experiments. #, significantly higher ($P < 0.01$) than the unstimulated (LPS) control; *, significantly lower ($P < 0.05$) than the untreated (hBD-3, LL-37) control; §, synergistic effect of the combination AC-PACs + LL-37; significantly lower ($P < 0.05$) than the sum of inhibitory values of each compounds taken individually.

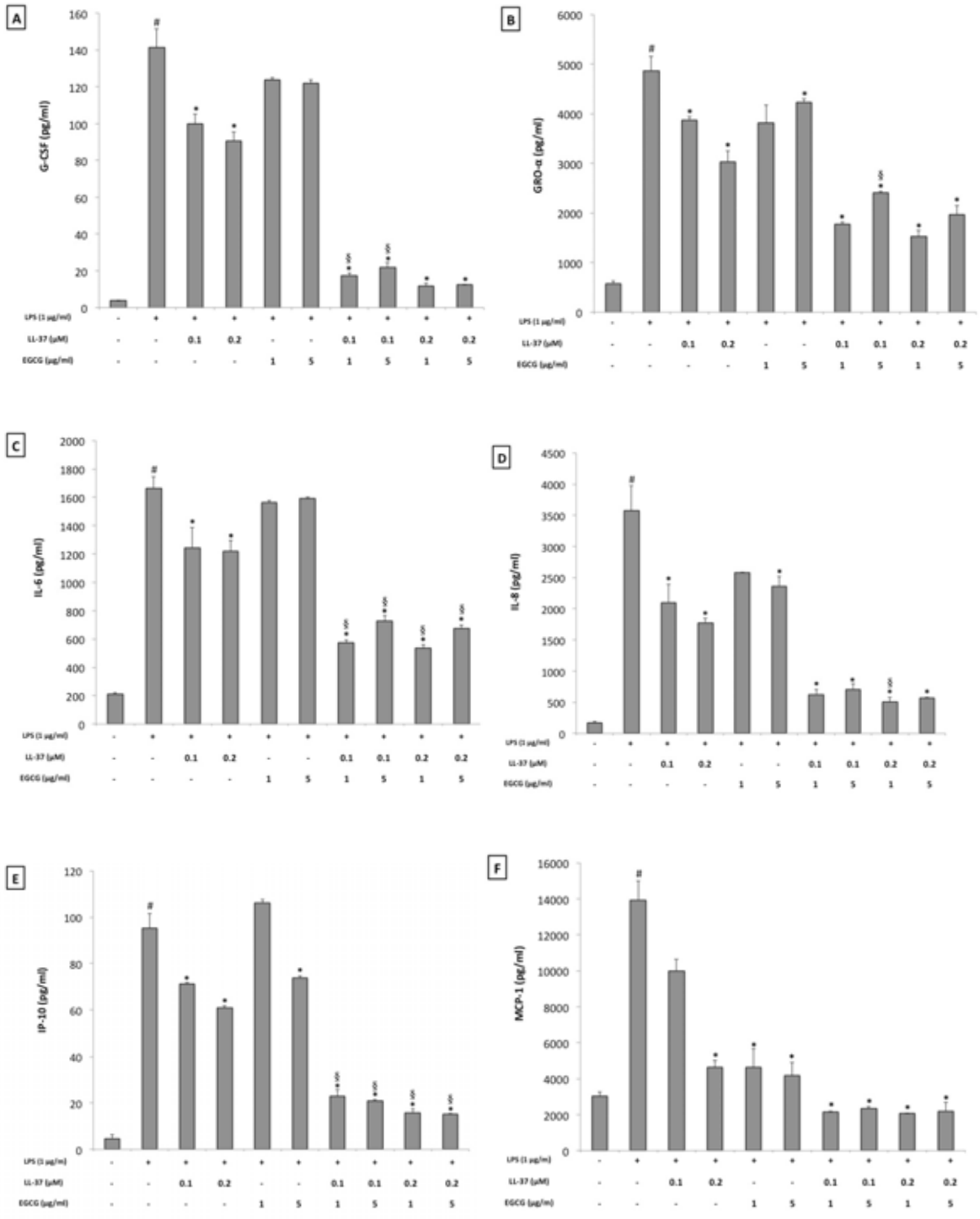

Figure 2: Effect of EGCG and LL-37, individually and in association, on the secretion of G-CFS (A), GRO-α (B), IL-6 (C), IL-8 (D), IP-10 (E) and MCP-1 (F) by the LPS-stimulated 3D co-culture model. Results are expressed as means ± standard deviations of triplicate assays from two independent experiments. #, significantly higher ($P < 0.01$) than the unstimulated (LPS) control; *, significantly lower ($P < 0.05$) than the untreated (hBD-3, LL-37) control; §, synergistic effect of the combination EGCG + LL-37; significantly lower ($P < 0.05$) than the sum of inhibitory values of each compounds taken individually.

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Considerações finais

CONSIDERAÇÕES FINAIS

Considerando as características multifatoriais das doenças periodontais e o elevado número de resistência bacteriana, busca-se novas terapias que possam contornar os problemas de resistência bacteriana e que atuem na modulação da resposta do hospedeiro.

Hoje em dia, muitas pesquisas têm sido desenvolvidas na busca por terapias alternativas aos antibióticos. Neste contexto, a utilização de compostos naturais surgem como alternativas devido ao baixo índice de resistência bacteriana. Dentre os compostos naturais, destacam-se os polifenóis provenientes do chá verde, o EGCG, e do cranberry, o AC-PACs, os quais possuem efeito antibacteriano e anti-inflamatório benéficos para a saúde[7, 10].

A associação de diferentes drogas possuem resultados satisfatórios na regulação da resposta imune do hospedeiro, pois é possível atingir diferentes microrganismos ao mesmo tempo e com baixas doses terapêuticas, as quais não irão gerar citotoxicidade nos tecidos[13].

No capítulo 1, pode-se concluir que o extrato do chá verde assim como seu principal polifenol o EGCG, além da comprovada ação antimicrobiana e anti-inflamatória demonstrada na literatura, foram capazes de induzir a produção de hBD-1 e hBD-2, assim como impedir a degradação dessas hBDs pelo *P. gingivalis*. Levando-se em consideração o importante papel destas defensinas na prevenção das infecções bacterianas, o extrato do chá verde e o EGCG surgem como promissores agentes terapêuticos das periodontites.

Já no estudo 2 foi possível observar as interações celulares que ocorrem entre as células epiteliais e fibroblastos, assim como o efeito sinérgico destes dois tipos celulares em resposta à um estímulo com LPS bacteriano. Portanto pode-se demonstrar a importância da utilização de modelos de co-cultura celular nos estudos in vitro, no intuito de melhor mimetizar as reações que ocorrem na cavidade bucal.

Pode-se observar também no estudo 2 que a hBD-3 e o LL-37, considerados importantes peptídeos antimicrobianos presentes na cavidade oral, são capazes de produzir um efeito sinérgico na diminuição da produção de citocinas pró-inflamatórias frente à um estímulo com LPS de *A. actinomycetemcomitans*, comprovando portanto a importância da associação de diferentes drogas terapêuticas que possam atuar em sinergismo como o caso da hBD-3 e o LL-37 e consequentemente prevenir os tecidos orais de possíveis destruição periodontais.

No estudo 3, o peptídeo LL-37 também mostrou efeitos sinérgicos em associação com o AC-PACs e o EGCG na redução da produção de citocinas pró-inflamatórias, portanto comprovou-se mais uma vez a importância da associação de diferentes drogas para obter melhores resultados terapêuticos com doses menores. Com isso o LL-37 assim como o AC-PACs e o EGCG surgem como importantes agentes terapêuticos.

Conclusão

CONCLUSÃO

Com os estudos apresentados aqui, conclui-se que os peptídeos antimicrobianos hBD-3 e LL-37, assim como o AC-PACs, que é uma importante proantocianidina do cranberry, o extrato do chá verde e seu principal polifenol, o EGCG mostraram efeitos benéficos na regulação da resposta imune. Os peptídeos assim como os compostos naturais mostraram efeito sinérgico na regulação da produção de citocinas pró-inflamatórias em resposta à uma agressão bacteriana, e o extrato do chá verde e o EGCG foram capazes de aumentar a produção de defensinas pelas próprias células do hospedeiro e consequentemente aumentam a barreira mecânica do organismo frente à uma agressão microbiana. Assim, os peptídeos antimicrobianos (hBD-3 e LL-37) e os compostos naturais surgem como terapias promissoras para o tratamento e prevenção das doenças periodontais.

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Apêndice

Apêndice 1: Material e Métodos

Protocolo do modelo em 3D de co-cultura de células epiteliais e fibroblastos

Cultura de células epiteliais e fibroblastos gengivais

Foi utilizado uma linhagem de células epiteliais gengivais humana denominada OBA-9, as quais foram gentilmente cedidas pela Prof. Dr. Márica Mayer (Departamento de Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, São Paulo, Brasil). As células epiteliais foram cultivadas em meio de cultura de queratinócito livre de soro (K-SFM; Life Technologies Inc.), contendo insulina, fatores de crescimento e 100 μg/mL de penicilina G- estreptomicina.

Foi utilizado fibroblastos gengivais humano denominado HGF-1 (ATCC ® CRL - 2014) os quais foram obtidos do banco americano de cultura celular (ATCC). Os fibroblastos foram cultivados em meio de cultura Dulbecco Eagle modificado (DMEM) suplementado com 4 mM de L-glutamina (Hyclone Laboratories, Logan, UT, EUA), 10 % de soro fetal bovino inativado (SFB) e 100 μg/ml de penicilina G - estreptomicina.

Ambas as células (HGF-1 e OBA-9) foram cultivadas em frascos de cultura de células de 75 cm^2 contendo 20ml de seus respectivos meio de cultura (K-SFM, DMEN – suplementados) e foram incubadas a 37°C em atmosfera contendo 5% de CO_2 até atingirem confluência .

Na fase experimental, as células epiteliais e os fibroblastos foram descongelados e cultivados rotineiramente em frascos de 75 cm^2 com 20 ml de meio de cultura e mantidos a 37°C em atmosfera contendo 5% de CO_2 em ambiente constantemente umidificado com substituição do meio a cada 2–3 dias.

Protocolo do modelo de co-cultura de células epiteliais e fibroblastos gengivais em 3D

Para o modelo de co-cultura, todos os reagentes foram colocados no gelo (DMEN-10x, Colágeno bovino tipo I, NaOH), pois a temperatura é um fator importante para a obtenção do gel de colágeno na presença dos fibroblastos gengivais.

Primeiramente foi preparada a solução de colágeno contendo 78% de colágeno bovino tipo I (95-98 % ; PureCol , Advanced BioMatrix , Tucson , AZ , EUA), 10% de DMEM (10X) (Sigma - Aldrich Canadá , Oakville , ON , Canadá) e 12% de NaOH para o ajuste ideal do pH à 7. Esta solução foi mantida no gelo durante todo o experimento.

Posteriormente as garrafas de HGF-1 com confluência em torno de 85% foram removidas da incubadora e a elas foram adicionados 5 ml de tripsina-EDTA a 0.05% (GibcoBRL , Grand Island , NY , EUA) e incubadas durante 5 minutos, no intuito de soltar as células do fundo da garrafas. Em seguida, a tripsina foi inativa pela adição de 10 ml de DMEN suplementado com 10% de SFB e foi realizado leve agitação das garrafas para uma completa liberação das células. A solução contendo os fibroblastos foi centrifugada a 1500 rpm durante 5 minutos e, em seguida os fibroblastos foram suspensos na solução de colágeno em uma concentração de $5x10^5$ células/ml. Esta suspensão foi então semeada em placas de cultura contendo 6 poços (2 ml/well, 2.5 mm de espessura do gel) (Sarstedt , Newton , NC , EUA). As placas de cultura foram em seguida incubadas a 37°C em aerobiose durante 2 horas para permitir a solidificação do gel de fibroblastos embebidos na matriz de colágeno. Após 2 horas de incubação foi adicionado 1ml de DMEN suplementado com 10% de SFB sobre o gel de colágeno, afim de evitar a desidratação do gel. A placa então foi incubada por mais 10 horas em atmosfera umidificada contendo 5% de CO_2 a 37°C.

Ao término de 10 horas de incubação, as garrafas contendo as células epiteliais com confluência maior que 80% foram removidas da incubadora e para o desprendimento das células do fundo da garrafa, 4 ml de Tripsina (TrypLE ™ expresso ; Life Technologies Inc.) foram adicionados e as garrafas foram incubadas a 37^oC por um período de 5 min. Em seguida a tripsina foi inativada através da adição de 4 ml de inibidor de tripsina a 0.3 mg/ml e foi realizado leve agitação das garrafas para uma completa liberação das células. A solução contendo as células epiteliais foram então levadas para centrifugação (1500 rpm durante 5 minutos), em seguida as células foram suspensas no meio de cultura K-SFM. As placas de cultura contendo o gel de fibroblastos foram então removidas da incubadora, o meio DMEN aspirado e 2 ml da OBA-9 em uma concentração de $1x10^6$ células/ml foram semeadas sobre o gel de fibroblastos. As placas foram então incubadas por mais 15 horas em atmosfera umidificada contendo 5% de CO_2 a 37°C. Após o período de incubação, o meio de cultura foi aspirado, as células foram estimuladas com os peptídeos (hBD-3 e LL-37) e os compostos naturais (AC-PACs e EGCG) 2 horas antes do estímulo com o LPS bacteriano. Em seguida as placas foram incubadas por 24 horas em atmosfera umidificada contendo 5% de CO_2 a 37°C. Após o período de incubação, o sobrenadante foi coletado e transferido para tubos de eppendorf. As amostras foram centrifugadas e os sobrenadantes aliquotados em novos tubos e armazenados à -20°C até o momento do processamento das amostras.

Anexo

ANEXO 1: Carta de envio de artigo – capítulo 2

FW: TR : Editor handles PEPTIDES-D-14-00096

De: **TELMA BEDRAN** (telmabedran@hotmail.com)
Enviada: quarta-feira, 26 de fevereiro de 2014 01:49:46
Para: TELMA BEDRAN (telmabedran@hotmail.com)

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> Objet : Editor handles PEPTIDES-D-14-00096
>
> Ms. Ref. No.: PEPTIDES-D-14-00096
> Title: 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
> Peptides
>
> Dear Dr. Daniel Grenier,
>
> Your submission entitled "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" will be handled by Editor Abba J. Kastin.
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