UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

MORGANA RODRIGUES GUIMARÃES

Efeitos do curcumin na modulação da inflamação e reabsorção óssea em dois modelos de doença periodontal em roedor.

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Tese apresentada ao Programa de Pós-Graduação em Odontologia; Área – Periodontia, da Faculdade de Odontologia da Universidade Estadual Paulista, para obtenção do título de doutora em Odontologia

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DADOS CURRICULARES

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Aos meus pais,

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SUMÁRIO

ABREVIATURAS

AP-1 – Activator protein 1
Bcl-2 - B-cell lymphoma 2

BSA – Bovine Serum Albumin

CCL_4 – Carbon tetrachloride

cDNA – complementary DNA

COX- (1, 2) - Cyclooxigenase (-1, -2)

CRP – C - reative protein

DNA – Deoxyribonucleic Acid

DNB - Dinitrobenzene sulfonic acid

ERK - Extracellular-regulated kinase

FBS – Fetal Bovine Serum

IL-1β – Interleukin 1 beta

IL(-1; -2; -4, -6, -8, -12, 17) – Interleukin (-1; -2; -4, -6, -8, -12, 17)

IFN-gamma - Interferon-gamma

IkB – Inhibitor of NF-Kb

IKK – IkB Kinase

INOS: Nitric oxide synthase

JNK – c-Jun N-terminal kinase

LPS – Lipopolysaccharide

MAPK - Mitogen-activated protein kinase

MMIF - Macrophage Migration Inhibitory Factor

MMP (-1, -2, -3, -9) - Matrix metalloproteinases (-1, -2, -3, -9)

MPO – Myeloperoxidase

NF-kB - Nuclear Factor kappa B

NOS - Nitrous oxide systems

PBS – Phosphate Buffer Solution

PGE_2 – Prostaglandina E_2

OPG –Osteoprotegerin

RANK - Receptor activator of NF-kB

RANKL - Receptor activator of NF-kB ligand

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcriptase – Polymerase Chain Reaction

STAT-3 - Signal transducer and activator of transcription 3

Th (1, 2) – Lymphocytes T helper (1, 2)

TLR – Toll like receptor

TNBS - Trinitrobenzene sulfonic acid

TNF-α – Tumor Necrosis Factor – alpha

TRAF6 – TNF Receptor Associated Factor 6

Resumo

Guimarães MR. Efeitos do curcumin sobre a modulação da inflamação e da reabsorção óssea em dois modelos de doença periodontal em roedor [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2010.

Curcumin, um polifenol derivado do tumérico (*Curcuma longa*) modula a atividade dos fatores de transcrição NF-κB e AP-1 e reduz a expressão de uma série de mediadores inflamatórios. Suas aplicações terapêuticas têm sido estudadas em uma variedade de condições, tais como colite, artrite reumatóide, câncer, psoríase; mas este é o primeiro estudo avaliando seus efeitos na doença periodontal in vivo. O inicio e progressão da doença periodontal ocorre como consequência da resposta do hospedeiro aos microorganismos do biofilme dental. Uma resposta imune imprópria induz à produção exagerada de citocinas inflamatórias e consequentemente à perda de inserção periodontal e reabsorção óssea. No estudo in vitro, uma linhagem celular de macrófagos de camundongo (Raw 264.7) foi estimulada com LPS de *E.coli* na presença e ausência de concentrações não citotóxicas de curcumin e de inibidores bioquímicos de NF-κB e p38 MAPK. RT-qPCR e ELISA foram usados para determinar a expressão de IL-6, TNF-alpha, COX-2/PGE_2 em nível de mRNA e proteína. O efeito do curcumin sobre a ativação das MAPKinases e NF-kB induzida por LPS foi avaliado por western-blot. No estudo in vivo, a doença periodontal experimental foi induzida em ratos pela colocação de ligaduras ao redor dos primeiros molares inferiores e injeção de LPS nos tecidos gengivais da região palatina entre os primeiros molares superiores (30 ug LPS, 3 vezes/semana por 2 semanas). Curcumin foi administrado aos animais diariamente por gavagem oral em duas doses (30 e 100 mg/Kg) durante as duas semanas do experimento, iniciando no dia anterior à indução da doença periodontal. Os animais controle receberam ligaduras e injeções de LPS, mas somente o veículo óleo de milho por gavagem. Os animais do grupo controle negativo não foram manipulados. RT-qPCR e ELISA foram usados para determinar a expressão de IL-6, TNF-alpha e PGE_2 nos tecidos gengivais. O processo inflamatório foi avaliado através da análise estereométrica, enquanto a modulação da sinalização p38 MAPK e NK-kB foram avaliadas por western-blot. A reabsorção óssea foi determinada por microtomografia computadorizada e por avaliação da área de superfície radicular exposta, enquanto a expressão de RANKL foi avaliada por imunoistoquímica. Os resultados in vitro mostraram que curcumin, mas não os inibidores bioquímicos de p38 MAPK e NF-kB,

reduziram os níveis de RNA mensageiro de IL-6, TNF-alpha e COX-2. Somente curcumin bloqueou completamente a expressão de IL-6 e TNF-alpha em nível protéico, enquanto tanto curcumin quanto os inibidores bioquímicos reduziram a expressão protéica de PGE_2. A ativação de NF-kB e de todas as três MAPKinases (ERK, JNK, p38) foram inibidas pelo curcumin. In vivo, em ambos os modelos de doença periodontal induzida, curcumin efetivamente inibiu a expressão gênica e inibiu de maneira dose-dependente a ativação de NF-kB nos tecidos gengivais. A ativação de p38 MAPK in vivo não foi afetada pelo curcumin. Os animais tratados com curcumin também apresentaram uma marcada redução no infiltrado inflamatório celular, aumento no conteúdo de colágeno e no número de células fibroblásticas. No entanto a reabsorção óssea não foi afetada pela administração de curcumin, que também não modulou de forma marcante a expressão de RANKL nos tecidos gengivais. Curcumin administrado via oral inibiu a resposta imune associada com a doença periodontal, sugerindo um potencial terapêutico nestas condições crônicas inflamatórias.

Palavras-chave: Curcumina; doenças periodontais; inflamação; sinalização intracelular.

Abstract

Guimarães MR. Effects of curcumin on modulation of inflammation and bone resorption in two models of induced periodontal disease in rodent [Tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2010.

Curcumin, a polyphenol derived from turmerics (*Curcuma longa*), has been shown to down regulate the activity of transcription factors NF-κB and AP-1 and to inhibit the expression the several inflammatory mediators. Its therapeutic applications have been studied in a variety of conditions, including as colitis, rheumatoid arthritis, cancer, psoriasis; but this is the first study to assess the effects of curcumin on periodontal disease in vivo. Periodontal disease initiation and progression occurs as a consequence of the host response to microorganisms of the dental biofilm. An improper immune response leads to overproduction of inflammatory cytokines and consequently periodontal attachment loss and bone resorption. In vitro study, we stimulated Raw 264.7 murine macrophages with *E.coli* LPS in the presence and absence of non-cytotoxic concentrations of curcumin and of the biochemical inhibitors of NF-kB and p38 MAPK. RT-qPCR and ELISA were used to determine the expression of IL-6, TNF-alpha and Cox-2/PGE_2 at mRNA and protein levels. Western-blot was used to assess the effect of curcumin on LPS-induced activation of MAPKinases and NF-kB. In the vivo study, experimental periodontal disease was induced in rats by placing cotton ligatures around both lower first molars and injecting LPS in the gingival tissues on the palatal aspect of upper first molars (30 ug LPS, 3 times/week for 2 weeks). Curcumin was given to the rats by daily oral gavage in two doses (30 and 100 mg/Kg) during the 2 week-duration of the experiment. Control animals received ligatures and injection of LPS but only the corn oil vehicle by gavage and negative control animals were untouched. RT-qPCR and ELISA were used to determine the expression of IL-6, TNF-alpha and PGE-2 synthase on the gingival tissues. The inflammatory status was evaluated by stereometric analysis, whereas modulation of p38 MAPK and NK-kB signaling was assessed by western blot. Alveolar bone resorption was assessed by computerized microtomography and macroscopic evaluation of the area of exposed root surface. Immunohistochemistry was used to detect the expression of RANKL in the gingival tissues. The in vitro study showed that curcumin, but not p38 and NF-kB

inhibitors, inhibited LPS-induced expression of IL-6, TNF-α and Cox-2 mRNA. Only curcumin completely abrogated expression of IL-6 and TNF-α at the protein level, whereas both curcumin and the biochemical inhibitors reduced expression of PGE_2 protein. LPS-induced activation of NF-kB and all three MAPKinases (ERK, JNK and p38) were inhibited by curcumin. In both experimental models of periodontal disease, curcumin effectively inhibited cytokine gene expression at mRNA and protein levels and dose-dependently inhibited activation of NF-kB in the gingival tissues. p38 MAPK activation was not inhibited by curcumin. Curcumin-treated animals also presented a marked reduction on the inflammatory cell infiltrate and increased collagen content and fibroblastic cell numbers. Bone resorption and RANKL expression were not affected by curcumin. The potent inhibitory effect of the immune response suggests that curcumin has therapeutic potential in this chronic inflammatoty condition.

Key-words: Curcumin; periodontal disease; inflammation, cell signaling

INTRODUÇÃO

Introdução

Curcumin é um polifenol extraído das raízes e do rizoma seco da planta *Curcuma Longa*, largamente utilizado como condimento e agente colorante, principalmente no sul e sudeste tropical da Ásia onde a planta é largamente cultivada [62]. Além do uso culinário, curcumin tem sido usado para tratar desordens biliares, hepáticas, artrite, diabetes e lesões de pele e câncer [11, 27, 46, 97, 112, 119, 122, 128, 130]. De fato, o potencial terapêutico do curcumin vem sendo intensamente pesquisado, como demonstra uma busca online na base de dados do National Institutes of Health (NIH) dos Estados Unidos (http://www.clinicaltrials.gov) que listava, em 2010, mais de 40 estudos clínicos cadastrados avaliando curcumin nas mais variadas condições incluindo diversos tipos de câncer (cólon, pulmão, linfoma, mama), psoríase, artrite, e até mesmo depressão.

Os mecanismos moleculares de ação do curcumin ainda são pouco conhecidos. Praticamente insolúvel em água e éter, curcumin pode ser dissolvido em etanol, dimetilsulfóxido (DMSO) e acetona. Sua estabilidade em meio aquoso melhora em pH alto (acima de 11.7), e embora alguns de seus componentes tenham se mostrado sensíveis à luz, os efeitos combinados de ar e luz não se mostraram deletérios [6, 40, 46].

Vários modelos animais [103, 114] ou estudos em humanos [77, 118] têm demonstrado uma virtual ausência de toxicidade do curcumin, mesmo quando administrado em doses elevadas. Três diferentes estudos clínicos fase I, indicaram que doses tão altas quanto 12 g por dia são bem toleradas [27, 35, 119]. Avaliações em ratos confirmaram uma ausência de toxicidade significante quando doses maiores que 5 g/Kg (o equivalente a 350 g de curcumin para um indivíduo de 70 Kg) foram administradas via oral [133]. Estudos pré-clínicos sistemáticos, fomentados pela Divisão de Prevenção do Instituto Nacional do Câncer nos Estados Unidos, não encontraram efeitos adversos do curcumin em ratos,

cachorros ou macacos que receberam doses maiores que 3.5 g/Kg/dia administradas por 3 meses ou mais [1]. Nenhum efeito adverso do curcumin foi observado em um estudo clínico com pacientes portadores de artrite reumatóide que utilizaram doses diárias de 1.2 a 2.1 g de curcumin via oral por 2 a 6 semanas [35]. Em pacientes com câncer de colo retal avançado, o tratamento com curcumin em doses até 3.6 g por até 4 meses foi bem tolerado [115.] Neste estudo, apenas dois casos de efeitos adversos do curcumin foram relatados: um paciente tratado com 0.45 g e outro tratado com 3.6 g de curcumin/dia desenvolveram diarréia, um mês e quatro meses após o inicio do tratamento, respectivamente.

A eficácia e segurança farmacológica têm feito do curcumin um candidato potencial para o tratamento de uma grande variedade de doenças [6]. Porém, a despeito de sua segurança e eficácia, problemas relacionados à biodisponibilidade tem sido um entrave. Pobre absorção gastrointestinal, rápida eliminação e pobre solubilidade aquosa são fatores que diminuem severamente sua biodisponibilidade [40], e de acordo com alguns estudos tais fatores independem da via de administração utilizada [6, 40, 115].

Algumas estratégias têm sido avaliadas com o objetivo de prolongar o tempo de circulação, aumentar a permeabilidade gastrointestinal e a resistência do fitoterápico à degradação por processos metabólicos. O uso de adjuvantes como piperine, um conhecido inibidor da metabolização hepática e intestinal, e de novas formulações como a sua inclusão em nanopartículas, tecnologia que tem emergido como uma solução proeminente para aumentar a biodisponibilidade de diversos agentes terapêuticos, a qual é particularmente indicada para agentes altamente hidrofóbicos como o curcumin, têm resultado em um aumento de até 154% em sua biodisponibilidade [18, 78, 118].

Este interesse no estudo dos efeitos do curcumin e de estratégias para melhorar sua biodisponibilidade se justifica pelas suas diversas atividades farmacológicas de

grande interesse em Saúde, incluindo propriedades antiinflamatórias, antimicrobianas, antivirais, antifúngicas, antioxidantes e cicatriciais. Curcumin pode também suprimir o início e a metástase tumoral em modelos experimentais in vivo [57, 58, 101] e agir como um agente antiproliferativo através da interrupção do ciclo celular, induzindo apoptose e a desintegração de estruturas mitóticas [29, 54, 81.] Devido a estas propriedades, curcumin tem sido avaliado em diversos estudos in vitro e in vivo no tratamento de doenças inflamatórias como artrite reumatóide, diabetes, doença de Crohn, pancreatite, psoríase e câncer. Um resumo dos principais achados destes trabalhos está presente na Tabela 1.

Tabela 1: Dose e efeito do curcumin nas doenças inflamatórias

Doença	**Dose**	**Efeito**	**Referência**
Doença Inflamatória Intestinal e colite			
Camundongo	0,25% dieta	Atenua colite induzida por DNB, reduz ativação de NF-KB, p-38, IL-1beta, MPO.	112
Camundongo	50 mg/Kg intra-gástrica	Diminui colite induzida por TNBS, reduz MPO, INOS.	24
Ratos	180 a 220 mg intra-peritoneal/oral	Bloqueou a ulceração gástrica, acelerou a cicatrização e atenuou atividade de MMP-9 e MMP-2.	125
Ratos	200 mg/kg; 400 mg/kg oral	Melhora histológica do fígado injuriado por CCL_4, redução nos níveis de TNF-alpha e IL-6.	43

Doença	Dose	Efeito	Referência
Ratos	200 mg; infusão intravenosa	Inibiu os níveis de mRNA de IL-6, TNF-ALPHA, INOS, bloqueou ativação de NF-κB, AP-1. Melhorou histologicamente a pancreatite e reduziu a infiltração de neutrófilos no pâncreas.	49
Artrite reumatóide			
In vitro	1; 10 µM	Inibiu a expressão de MMPs induzidas por MMIF em fibroblasto sinoviais da artrite reumatóide.	97
In vitro	0 a 100 µM	Curcumin induziu apoptose e reduziu a expressão de PGE-2 de fibroblasto sinoviais.	100
In vitro	50 µM	Inibição da expressão de COX-2, MMP-9 e colágeno tipo II em condrócitos estimulados por IL-1beta e TNF-ALPHA.	113
Ratos	30 mg/kg oral	Reduziu os níveis séricos de Gp A72 , importante marcador da artrite reumatóide, e a inflamação na pata de ratos com artrite.	65
Ratos	100 mg/kg oral	Redução de IL-1beta e CRP induzidos por adjuvante (Freund's).	11

Doença	Dose	Efeito	Referência
Diabetes e desordens metabólicas			
Ratos	0,1%, 0,25%, 0,5% dieta	Diminui o nível de colesterol no soro e fígado.	107
Ratos e camundongos	40 mg/kg oral	Favorece a cicatrização em ratos diabéticos e camundongos genética-mente diabéticos.	119
Ratos	80 mg/kg oral	Aumenta o nível de HDL e diminui os níveis de lipídio no soro e fígado.	99
Pacientes	5mg dieta	Menores níveis de glicose no sangue.	122
Câncer			
Pacientes: Leucoplasia oral, metaplasia intestinal do estomago.	1-12g/dia, cápsulas via oral	Melhora histológica em 7 pacientes, 2 desenvolveram malignidade.	27
Ratos	500ppm ou 0,5g/kg dieta	Curcumin causou uma redução significante na freqüência de carcinoma de língua induzido por 4-nitroquinolone 1-oxido (4-NQO).	128

A base molecular dos efeitos biológicos do curcumin é pouco conhecida, mas parece estar relacionada a ação sobre uma série de alvos incluindo fatores de transcrição, moléculas de adesão, genes relacionados a apoptose e angiogênese e moléculas de sinalização intracelular [2, 40].

Muitos dos seus efeitos biológicos envolvem processos que são dependentes de NF-κB; o que faz deste fator de transcrição um alvo preferencial da ação do curcumin. De fato a inibição de NF-kB por curcumin é um achado consistente em diversos estudos [4, 49, 117]. A ativação de NF-κB é crucial para imunidade inata e adaptativa e tem um papel importante na inflamação, doenças auto-imunes e câncer [20, 63]. Ativado por uma variedade de estímulos incluindo citocinas, mitógenos, carcinógenos, agentes quimioterápicos, estresse químico e outros estímulos inflamatórios, NF-kB está associado à regulação da expressão de aproximadamente 200 genes que estão envolvidos na inflamação, transformação celular, proliferação, angiogênese e invasão celular [2].

A inibição de NF-κB pelo curcumin resulta na redução da expressão gênica de diversas citocinas inflamatórias (TNF-alfa, IL-1, IL-6, IL-12) bem como de enzimas pró-inflamatórias (COX-2, MMPs e NOS) [20, 36, 86]. Em células mielóides humanas, curcumin mostrou um efeito supressivo sobre a ativação de NF-κB induzida por TNF-alfa, éster de forbol e peróxido de hidrogênio [120]. Experimentos com macrófagos esplênicos de camundongos demonstraram que curcumin pode bloquear a ativação de TLR2 por LPS, a qual resultaria na ativação de NF-κB [84]. Curcumin basicamente inibe a transativação de NF-κB devido à supressão sequencial da fosforilação e degradação de IκB e subseqüente fosforilação de p65, inibindo a translocação nuclear da subunidade p65 de NF-κB [4].

A habilidade do curcumin em modular a via de sinalização MAPK também parece contribuir para a inibição da inflamação. As MAPKs são um grupo heterogêneo de enzimas responsáveis pela transdução de diversos sinais extracelulares, e promovem a fosforilação dos aminoácidos treonina e serina em múltipos substratos protéicos, incluindo outras enzimas, fatores de transcrição e proteínas ligantes ao RNA. Entre os

sinais externos capazes de ativar estas enzimas, estão estresse (radiação UV, alterações na pressão osmótica), citocinas e mediadores inflamatórios endógenos, LPS e outros antígenos microbianos e fatores de crescimento. Assim, além de exercerem papéis relevantes no desenvolvimento e homeostasia, um grande número de evidências pré-clinicas e clínicas confirmam seu papel crítico na inflamação e resposta imune e, consequentemente, no desenvolvimento de doenças inflamatórias [21].

Salh et al. [112] relataram que a administração via oral de curcumin (50mg/kg-100mg/kg) atenuou a colite experimental em ratos através de uma redução na atividade de p38 MAPK [112]. Em um estudo com células endoteliais humanas curcumin reduziu a fosforilação de p38 e JNK induzida por 10 ng/mL de TNF-alfa, ao mesmo tempo em que reduziu a expressão de espécies de oxigênio reativo intracelular (ROS) [71]. Os autores sugerem que a atenuação da resposta inflamatória por células endoteliais e monócitos pelo curcumin pode representar um efeito anti-angiogênico através da modulação não apenas de NF-κB, mas também de p38 e JNK MAPKinases.

Estes efeitos sobre as MAPKinases indicam que o curcumin é um potente inibidor dos fatores de transcrição AP-1, além de NF-κB, reduzindo a expressão de mediadores inflamatórios reconhecidamente envolvidos na osteoclastogênese; assim seu efeito sobre a modulação de doenças osteolíticas também tem sido avaliado. Ozaki et al. [98] demostraram que curcumin estimula a apoptose de osteoclastos de maneira dose-dependente e inibe dramaticamente a reabsorção óssea [98]. Estes autores utilizaram osteoclastos de coelhos, e verificaram que o tratamento com 10 μM de curcumin por 12 horas produziu alterações morfológicas indicativas de apoptose, como condensação da cromatina e fragmentação nuclear. Como NF-kB é um alvo preferencial do curcumin, estes dados sugerem a importância deste fator de transcrição sobre a sobrevivência de osteoclastos. Além de induzir apoptose de osteoclastos, os mesmos autores verificaram

que curcumin inibiu a diferenciação e atividade de osteoclastos de forma dose-dependente [98]. Estes efeitos foram acompanhados pela inibição da fosforilação de IκB e, consequentemente, da ativação de NF-κB.

A modulação do turnover do tecido ósseo pelo curcumin foi avaliada em modelo animal de osteoporose pós-menopausa [42]. Doses maiores de curcumin (750mg/kg) produziram um aumento no tamanho do fêmur. O curcumin também aumentou, de forma significativa e dose-dependente, a resistência tensional do fêmur à fratura experimental e reduziu a atividade de reabsorção do tecido ósseo, avaliada pela medição dos níveis séricos dos marcadores C-telepeptideo e osteocalcina, de forma menos potente do que no grupo controle positivo (tratado com etidronato + ovarectomia), porém significativamente melhores do que o grupo controle negativo (ovariectomia) [42]. Curcumin também inibiu a osteoclastogênese em modelo animal de diabetes induzida por estreptozotocina [52]. Neste estudo, a administração de curcumin como suplemento dietético reverteu os níveis de atividade e expressão de fosfatase ácida resistente ao tartarato (TRAP) e cathepsina K aos níveis de animais-controle não diabéticos. A análise histoquímica mostrou que a ingestão de curcumin produziu uma redução no número de células TRAP-positivas no fêmur distal das ratas diabéticas a níveis comparáveis ao de animais controle não diabéticos. Segundo os autores, ainda de acordo com os resultados o mecanismo de ação do curcumin sobre a osteoclastogênese parece envolver a modulação da expressão de c-fos e c-jun, proteínas que compõe o do fator de transcrição heterodimérico AP-1. Estes resultados indicam que curcumin tem efeito significativo na modulação da reabsorção óssea, despertando o interesse de diversos grupos de pesquisa na avaliação do curcumin em doenças ósseas, como artrite reumatóide, doença de Paget e osteoporose [42, 52].

A doença periodontal é uma infecção crônica do periodonto que compromete ambos, tecido mineralizado e tecido mole ao redor dos dentes. A progressão da doença está associada com a inflamação crônica dos tecidos moles, degradação das fibras colágenas bem como reabsorção óssea. Citocinas inflamatórias, bem como os fatores de transcrição NF-kB e AP-1, além das MAPKinases, todos fatores modulados pelo curcumin, tem importante papel na patogênese das doenças periodontais [40, 54, 73].

Não há dúvidas sobre a necessidade do estímulo bacteriano para o inicio da doença periodontal [66, 79]. Em resposta aos agentes bacterianos, reações defensivas do hospedeiro são ativadas para prevenir o avanço da infecção nos tecidos gengivais. Esta resposta do hospedeiro aos agentes infecciosos tem como dano colateral a destruição dos tecidos conjuntivo e ósseo devido à ação das citocinas inflamatórias derivadas do hospedeiro. Embora estas reações imune-inflamatórias sejam essenciais para a defesa do hospedeiro contra a infecção bacteriana, a reação excessiva e prolongada é prejudicial à função dos tecidos periodontais. A modulação da resposta imune-inflamatória, além do controle da infecção, é uma estratégia terapêutica ativamente pesquisada em Periodontia [126] e em outras condições associadas à inflamação crônica como a artrite reumatóide [72, 74, 75].

Neste contexto, as citocinas têm um papel fundamental na modulação dos sistemas inflamatórios. Elas são produzidas não somente por células imunes como linfócitos, monócitos, macrófagos e granulócitos, mas também por células epiteliais, células endoteliais e fibroblastos [126]. Em lesões inflamatórias periodontais as citocinas são produzidas localmente, porém o aumento de níveis séricos de citocinas inflamatórias na doença periodontal tem sido associado a potenciais efeitos deletérios sistêmicos, como o agravamento do processo arteriosclerótico [19, 90, 108].

Dentre as diversas citocinas inflamatórias, a produção de IL-6 e TNF-alfa nos tecidos periodontais tem sido amplamente estudada. Um dos importantes efeitos de TNF-alfa é estimular fibroblastos a produzir metaloproteinases que degradam componentes da matriz extracelular na artrite reumatóide [96] e periodontite [16]. Adicionalmente, TNF-alfa induz a reabsorção óssea aumentando a maturação e atividade dos osteoclastos [102]. Outras moléculas inflamatórias como PGE2 [91] e IL-8 [127] também são produzidas por fibroblastos gengivais humanos estimulados com IL-1beta e TNF-alfa . Estes achados sugerem que quantidades excessivas de TNF-alfa produzidas nos tecidos periodontais causam secreção de outros mediadores inflamatórios endógenos resultando na destruição tecidual e inflamação crônica.

Elevados níveis de IL-6 também são produzidos localmente em pacientes com periodontite crônica [48]. Isso pode ser resultado de uma infiltração prolongada de linfócitos no interior dos tecidos periodontais, produzindo imunoglobulinas contra os antígenos bacterianos. TNF-alfa e IL-6 estimulam a diferenciação de osteoclastos de maneira sinérgica e regulam a osteoclastogênese não somente de forma indireta, estimulando células estromais a produzirem RANKL, mas agindo diretamente sobre os osteoclastos e seus precursores [51, 61]. TNF-alfa aumenta a expressão de RNAm de RANK por células precursoras de osteoclastos e, sinergicamente com RANKL, estimula a formação de osteoclastos resultantes da fusão destas células precursoras [51].

Outro mediador importante no desenvolvimento da doença periodontal são as cicloxigenases. As enzimas cicloxigenase COX-1 e COX-2 catalisam a conversão do ácido araquidônico em prostaglandinas (PGs). COX-1 é uma enzima constitutiva normalmente expressa em uma variedade de tecidos e órgãos induzindo a formação de PGs com funções fisiológicas. COX-2 é uma enzima induzível de baixa expressão constitutiva, estimulada principalmente por mediadores pró-inflamatórios, levando à

formação de PGs envolvidas na periodontite e em outros processos patológicos como edema, artrite reumatóide, periodontite, hiperalgesia e febre [88, 104, 105] como indicam os resultados de estudos utilizando inibidores específicos de COX-2 [8, 50, 55].

As PGs, em especial PGE_2, podem estimular a reabsorção óssea por meio da indução do aumento tanto do número quanto da atividade dos osteoclastos [41, 93, 123]. Hormônios e citocinas envolvidas na reabsorção óssea podem estimular a expressão de COX-2 e a síntese de PGE_2 [93], sugerindo que o eixo COX-2/PGE_2 pode mediar a ação de alguns destes fatores associados à reabsorção óssea. Além disso, secreção elevada de PGE_2 está relacionada à perda óssea característica de algumas doenças inflamatórias como periodontite e artrite [95]. Especificamente na doença periodontal, PGE_2 tem sido constantemente correlacionada com inflamação e reabsorção óssea [12, 95]. Seus níveis nos tecidos gengivais e no fluido gengival crevicular são significativamente elevados em pacientes periodontalmente doentes [39, 95].

É importante notar que TNF-alfa e IL-6, assim como COX-2, ativam uma cascata de sinais intracelulares que incluem ativação dos membros da família MAPK (ERK, JNK, p38). As MAPK ativam diversos fatores de transcrição, incluindo AP-1, os quais induzem a transcrição de múltiplos genes-alvo, incluindo diversas citocinas pró-inflamatórias [13]. Além disso, ativadores *upstream* das MAPKinases, como TRAF6, também participam da ativação da via do NF-kB (Figura 1).

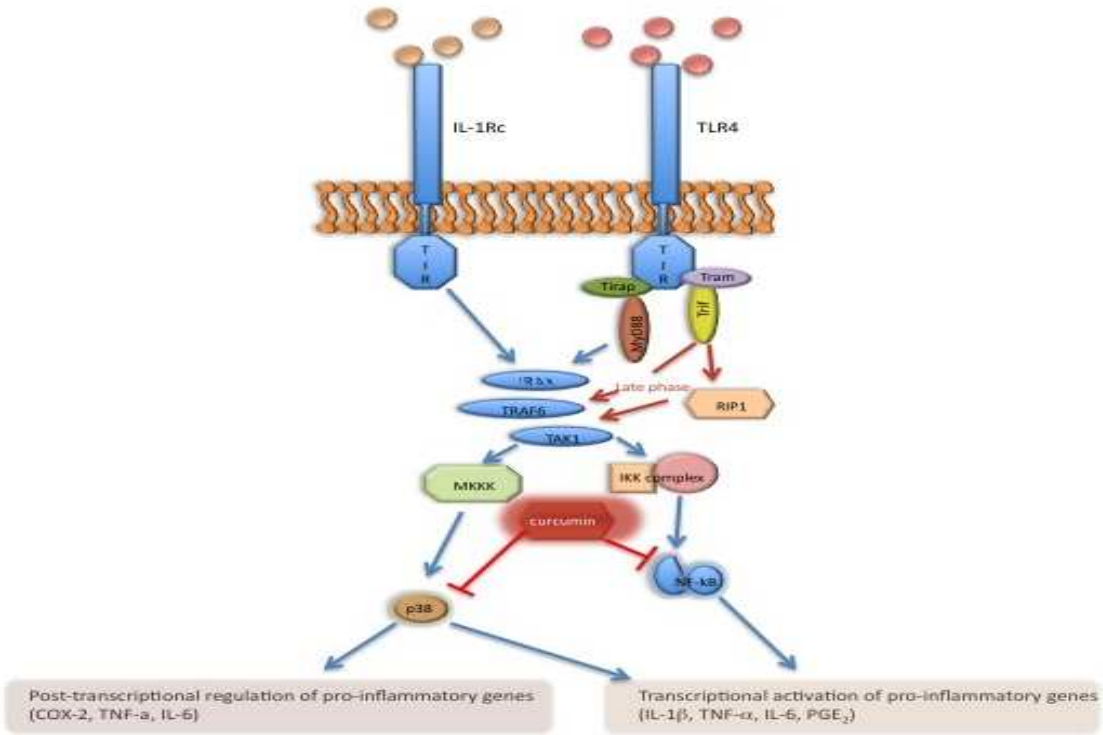

FIGURA 1: Modelo ilustrativo da regulação da transcrição de genes pró-inflamatórios pelos fatores de transcrição após estímulos externos. O papel inibitório do curcumin sobre p-38 e NF-κB bloqueando a ativação de mediadores inflamatórios, de acordo com o relatado na literatura, também está ilustrado (Figura modificada de Carlos Rossa Junior).

Especificamente na doença periodontal, a despeito da grande quantidade de informações disponíveis sobre a regulação e expressão de citocinas inflamatórias, há somente poucos estudos sobre a ativação das vias de sinalização ativadas in vivo. NF-κB tem mostrado estar associado com a severidade aumentada da doença periodontal [67]. Lipopolissacarídeo (LPS) bacteriano e citocinas pró-inflamatórias, todos presentes em grandes quantidades nos tecidos periodontais doentes, podem também ativar NF-κB. Imunohistoquimica para NF-κB (p50/p65) em tecido humano periodontalmente doente indicou uma expressão aumentada nos sítios de inflamação comparados com os sítios

saudáveis [5]. Com relação às MAPK, níveis de expressão dos três membros desta família são encontrados nos tecidos periodontais, embora estes níveis possam diferir dependendo do tipo celular e do grau de inflamação [72].

Diferenças interessantes na ativação das vias de sinalização foram encontradas em dois diferentes modelos de doença periodontal experimentalmente induzidas [45]. Em ambos, modelo de injeção de LPS e modelo de ligadura, p38 e ERK MAPkinases, bem como NF-κB foram ativadas, mas com cinéticas diferentes. Evidência in vitro para a relevância de p38 MAPK para a doença periodontal é primariamente derivada de estudos demonstrando o importante papel desta via de sinalização para a regulação da expressão de citocinas inflamatórias que são relevantes para o processo de doença [74]. As citocinas direta ou indiretamente reguladas por p38 MAPK incluem IL-1beta, IL-4, IL-6, IFN-gama, TNF-alfa, NO, PGE_2, MMP-13, RANKL em vários tipos celulares e associados com a resposta imune inata e adaptativa [32, 34, 85, 110, 131].

Diante do importante papel das citocinas inflamatórias e das vias de sinalização MAPKinases e NF-κB para o inicio e progressão da doença periodontal bem como para o desenvolvimento de outras doenças de natureza inflamatória, estratégias terapêuticas incluindo redução da produção e atividade de citocinas e bloqueio das vias de sinalização intracelular têm sido um interessante e promissor campo de estudo. Neste contexto, a utilização de produtos naturais com virtual ausência de efeitos secundários e com propriedades imuno-modulatórias, pode oferecer potencial significante no tratamento das doenças periodontais. Embora inúmeros trabalhos tenham avaliado os efeitos do curcumin no tratamento de diferentes doenças inflamatórias, seus efeitos sobre a modulação da doença periodontal ainda não foram explorados. **Dessa forma, este estudo se propõe a avaliar, pela primeira vez, os efeitos do curcumin sobre a evolução da doença periodontal experimentalmente induzida em ratos.**

PROPOSIÇÃO

Proposição

A hipótese deste trabalho é que a administração do curcumin poderia reduzir a severidade da destruição do tecido conjuntivo e a reabsorção do osso alveolar associadas à doença periodontal experimental através da modulação das vias de sinalização intracelular necessárias para expressão e ativação das citocinas inflamatórias. Este efeito poderia ainda ser influenciado pelo modelo experimental utilizado (ligadura ou LPS) devido às diferenças na resposta inflamatória induzida por cada modelo. Para avaliar esta hipótese, os objetivos específicos deste estudo foram:

1- Analisar o efeito do curcumin sobre a modulação das vias de sinalização e expressão de genes inflamatórios em uma população de células da resposta imune inata (macrófagos) in vitro.

2- Avaliar o efeito da administração sistêmica de curcumin sobre a resposta inflamatória e a modulação das vias de sinalização intracelular durante o curso de dois modelos de indução de doença periodontal experimental em ratos.

3- Avaliar o efeito do curcumin sobre a reabsorção do osso alveolar associada à doença periodontal induzida por dois modelos experimentais em ratos.

CAPÍTULO 1

Curcumin inhibits LPS-induced MAPK and NF-KB signaling, abrogating pro-inflammatory cytokine expression by RAW 264.7 macrophages

Running title: Curcumin inhibits LPS-induced cytokine expression

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Summary

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Background and purpose: Curcumin is the active compound in the extract of *Curcuma longa* rhizomes. It has been shown to have anti-inflammatory properties that are associated with inhibition of various signaling pathways. Considering that NF-kB and MAPKinases are involved in the expression of IL-6, TNF-α and PGE_2 and their important role on chronic inflammatory diseases, the aim was to evaluate the effect of curcumin on LPS-induced expression of these cytokines in macrophages.

Experimental approach: RAW 264.7 murine macrophages were stimulated with *E.coli* LPS in the presence and absence of non-cytotoxic concentrations of curcumin and of the biochemical inhibitors of NF-kB and p38 MAPK. RT-qPCR and ELISA were used to determine the expression of IL-6, TNF-α and Cox-2/PGE_2 at mRNA and protein levels. Western-blot was used to assess the effect of curcumin on LPS-induced activation of MAP Kinases and NF-kB.

Key results: Curcumin, but not p38 and NF-kB inhibitors, inhibited LPS-induced expression of IL-6, TNF-α and Cox-2 mRNA. Only curcumin completely abrogated expression of IL-6 and TNF-α at the protein level, whereas both curcumin and the biochemical inhibitors reduced expression of PGE_2 protein. LPS-induced activation of NF-kB and all three MAPKinases (ERK, JNK and p38) were inhibited by curcumin.

Conclusions and implications: Curcumin has therapeutic potential for host modulation in chronic diseases associated with innate immune signaling, such as periodontal diseases and infections of the lower intestinal tract. This is the first study to show that curcumin affects LPS-induced activation of NF-kB and all three MAPKinases in macrophages.

Keywords: Curcumin, innate immunity, cell signaling, cytokines, NF-kB, MAPK

Introduction

Macrophages, originated from circulating blood monocytes, are involved in the maintenance of homeostasis in normal tissue turnover, acting as scavengers to eliminate apoptotic and senescent cells and debris through their phagocytic function. Although this is a primary function of these cells, macrophages also play vital roles in inflammation and repair of injured tissue, by the production of a large number of cytokines, chemokines and growth factors that recruit and activate a variety of other cell types to the injured tissue. Thus, macrophages also play a fundamental role in the innate and adaptive immune responses and are involved in the pathogenesis of various diseases characterized by chronic inflammation, including atherosclerosis, rheumatoid arthritis, inflammatory bowel disease and periodontitis (Hunter *et al.*, 2009).

Interleukin (IL)-6 and Tumor necrosis factor (TNF)-alpha are two inflammatory cytokines that are produced by macrophages and other cells (T lymphocytes, fibroblasts, osteoblasts) and play important roles on the pathogenesis of various chronic diseases, including insulin-dependent diabetes mellitus (Campbell *et al.*, 1991; Ishihara *et al.*, 2002; Pilstrom *et al.*, 1995; Rabinovitch *et al.*, 1998), inflammatory bowel disease (Stevens *et al.*, 1992), ulcerative colitis (Reinecker *et al.*, 1993), arthritis (Feldmann *et al.*, 1991; Feldmann *et al.*, 2001), periodontitis, systemic lupus erythematosus and Crohn's disease (Cronstein, 2007; Gabay, 2006). Rheumatoid arthritis and periodontitis are two conditions characterized by chronic inflammation and increased bone resorption. IL-6 and TNF-α can stimulate osteoclastogenesis directly (Kwan Tat *et al.*, 2004) and indirectly by inducing the production of receptor-activator of nuclear factor kappa B ligand (RANKL) by other cells (osteoblasts, fibroblasts and T lymphocytes) and acting synergistically with RANKL on osteoclasts. Defined as a potent bone-resorbing factor, TNF-α is well known for its capacity to stimulate osteoclastic bone

resorption in vitro (Thomson *et al.*, 1987) and in vivo (Konig *et al.*, 1988). IL-6, also considered a bone-resorptive agent, has been shown to stimulate osteoclast activity and bone resorption by an indirect mechanism, increasing interactions between osteoblasts and osteoclasts (Gabay, 2006). Therapeutic strategies aimed at inhibition of IL-6 or TNF-α to reduce bone resorption associated with periodontitis, arthritis, orthopedic implant loosening and other forms of chronic inflammatory osteolysis (Bingham, 2002) including post-menopausal osteoporosis have been actively evaluated in clinical trials (Charatcharoenwitthaya *et al.*, 2007; Galarraga *et al.*, 2009; Genovese *et al.*, 2008).

However, instead of targeting the inhibition of a single cytokine specifically, another interesting strategy for modulating the host response is the prevention of cell activation by changing the way cells sense and respond to external signals. Cells perceive and adapt to diverse changes in the extracellular environment, including the presence of inflammatory cytokines that interact with membrane-bound receptors and induce changes in a multitude of intracellular proteins that transmit the external 'signal' to the nucleus of the cell where the appropriate response (e.g., proliferation, cell death, expression of various genes) will be initiated. These intracellular signal transduction pathways are activated not only by cytokines, but also by other factors, such as microbial-associated molecular patterns (e.g., bacterial lipopolysaccharide, LPS) or environmental stress. Since there are a relatively limited number of signaling pathways involved in the expression of many inflammatory genes in response to diverse external stimuli, modulation of these pathways can affect the expression of various cytokines, effectively changing the cytokine network associated with chronic inflammatory conditions. Information on the activation status of intracellular signaling pathways involved in the expression of inflammatory mediators is crucial for the evaluation of therapeutic strategies based on the modulation of the host response.

Among the intracellular signaling pathways involved in cytokine production, the transcription factor NF-kB, mitogen-activated protein kinase (MAPK) and the suppressors of cytokine signaling (SOCS), were shown to play fundamental roles. NF-kB is one of the most important inducible transcription factors in mammals and has been shown to play a pivotal role in the mammalian innate immune response (Hoffmann *et al.*, 1999) and chronic inflammatory conditions (Ghosh *et al.*, 1998). MAPKs, which include JNK, ERK, and p38, are well known stress-activated kinases implicated in the regulation of many genes, particularly those of the inflammatory and immune response (Kim *et al.*, 2007). The SOCS family consists of eight proteins (SOCS-1 to -7, and cytokine-inducible SH2-domain-containing protein) that are produced in response to a diverse range of cytokines and growth factors and acts to attenuate signal transduction as part of a negative feedback loop to inhibit the response to subsequent stimuli (Garlet et al. 2006; Alexandre WS, 2004; Naka T et al 2005; Rakesh K 2005). Thus, modulation of these cytokine signaling pathways can have a profound effect in the treatment of various inflammatory conditions.

Curcumin, a polyphenol derived from a plant, *Curcuma longa*, has potent anti-oxidant and anti-inflammatory activities. This compound has been shown to inhibit in vitro the expression of a sequence of inflammatory cytokines such as TNF-α, IL-8, IL-1, IL-6, MMP-9, NO, COX-2 in various cell types (Brouet *et al.*, 1995; Lantz *et al.*, 2005; Saja *et al.*, 2007; Shakibaei *et al.*, 2007; Surh, 2002; Swarnakar *et al.*, 2005), even though the information on the macrophage-specific effects of curcumin are relatively scarse. Because the overexpression of these inflammatory mediators is closely linked to inflammatory disease development, many studies have evaluated the effect in vivo of curcumin in the treatment of these diseases, such as cancer (Garcea *et al.*, 2004; Sharma *et al.*, 2004; Sharma *et al.*, 2001), rheumatoid arthritis (Deodhar *et al.*, 1980), Crohn`s

disease (Holt *et al.*, 2005), inflammatory bowel disease (Bundy *et al.*, 2004), colitis (Hanai *et al.*, 2006) and psoriasis (Heng *et al.*, 2000). According to these reports, curcumin substantially attenuated the clinical symptoms and decreased the levels of main biologic markers of these pathologies.

The downregulation of inflammatory mediators by curcumin is associated with the modulation of the transcription factors NF-kB and AP-1. AP-1 is a heterodimeric transcription factor that is primarily activated by MAPKinase signaling pathway (Bharti *et al.*, 2004). Curcumin has been shown to inhibit NF-kB, MAPK and other signaling pathways involving other protein tyrosine kinases as well as serine/threonine kinases, such as JAK/STAT and PKC (Aggarwal *et al.*, 2003; Bharti *et al.*, 2003; Bharti *et al.*, 2004; Rafiee *et al.*, 2009). This modulation of signaling pathways varies according to the external stimulus and cell type.

The cellular targets of curcumin in the immune system are poorly understood. Some groups have shown its suppressive effect on the proliferation of splenic lymphocytes (Gao *et al.*, 2004); the inhibition of LPS-induced immunoestimulation of murine dendritic cells (Kim *et al.*, 2005) and macrophages (Brouet *et al.*, 1995; Chen *et al.*, 2008).

In this study, we analyzed the effects of curcumin on the modulation of macrophage response to bacterial LPS. We demonstrated that curcumin suppressed LPS-induced TNF-α, IL-6, PGE-2 expression and this effect is paralleled by inhibition of the activation of NF-kB, p38 and ERK and increase of socs-1-3.

Materials and Methods

Cell line and materials

The murine macrophage cell line RAW 264.7 (ATCC #TIB-71) was cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere at 37°C and 5% CO_2. LPS from *Escherichia coli* (serotype O55:B8) and curcumin (C1386) were purchased from Sigma. Biochemical inhibitors for p38 (SB203580), NF-kB (Bay 11-7082) were from Calbiochem. *E. coli* LPS was diluted in PBS (pH 7.4) at 1mg/ml and used at a final concentration of 1 µg/ml, curcumin and biochemical inhibitors were diluted in DMSO. Rabbit polyclonal antibodies against phosphorylated forms of p38 MAP kinases, NF-kB (p65), ERK, SOCS-1-3 and to the housekeeping beta-actin were from Cell Signaling, as well as the secondary HRP-conjugated antibodies.

Cell viability

The effect of curcumin and NF-kB inhibitor on cell viability was determined using a commercial kit (Cell Titer 96 Aqueous; Promega Corp.) according to the manufacturer's instructions. This kit evaluates cell viability by means of reduction of a tetrazolium containing solution to formazan by dehydrogenase enzymes present in metabolically active, viable cells. Briefly, 1 x 10^5 cells were plated in each well of 96-well plates, allowed to attach for 18h, washed once with phosphate-buffered saline (PBS) and then deinduced in culture medium with reduced concentration (0.3%) of FBS. Curcumin was added to the culture medium in various concentrations for a dose-response experiment and the cells incubated for 24 h. Controls were represented by the same volume of the DMSO vehicle and also by different concentrations of the NF-kB inhibitor (Bay 11-7082). 20 µl of reagent containing the tetrazolium salt (MTS) was added to each well and incubated for 1 and 2 hours and the results obtained by measuring the absorbance at 490 nm on a microplate reader (Bio-Rad Inc., model 550).

The relative number of viable cells in the wells treated with curcumin or Bay 11-7082 was estimated in relation to the vehicle-treated control wells.

Evaluation of cytokine gene expression at the mRNA level (RT-qPCR)

A total of 3 x 10^5 cells were grown for 24 h in each well of six-well plates, de-induced by incubation for 8 h in culture medium containing 0.3% fetal bovine serum and stimulated with *E. coli* lipopolysaccharide for 24 h, both with and without a 30 min pretreatment with 5 µM or 10 µM of curcumin. Other wells were treated with 10 µM of the p38 MAPK inhibitor (SB203580) or 10 µM of the NF-kB inhibitor (Bay 11-7082). Control wells were treated with the same volume of the DMSO vehicle used to dilute these compounds. Total RNA was isolated from the cells with Trizol reagent (Invitrogen Corp.) according to the manufacturer's instructions. The RNA was quantitated by spectrophotometry and 500 ng were reverse-transcribed into cDNA. The relative abundance of the transcripts of the candidate inflammatory genes were measured by real-time reverse transcription-PCR (RT-PCR) using Taqman chemistry and pre-designed sets of primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) on a StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate on a final reaction volume of 30 µL that included Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) for each target gene: tnf-α (tumor necrosis factor alpha), NM_013693; il-6 (interleukin 6), NM031168; Ptgs-2 (prostaglandin-endoperoxide synthase-2), NM011198; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), NM008084;,and cDNA template (corresponding to 30 ng of total RNA). Optimized thermal cycling conditions were: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 1 min. For each sample, analyses of gene expression were performed in duplicate. Three independent

experiments were performed. To normalize the amount of total RNA present in each reaction, the expression of GAPDH, which was not altered by the experimental conditions, was used as a housekeeping gene. To compare the expression levels among different samples, the relative expression level of the genes was calculated using the comparative ΔCT method using the thermocycler's software.

Determination of cytokine gene expression at the protein level (ELISA)

Cell culture supernatants were collected from the same wells from which total RNA was harvested immediately thereafter. In brief, cells were stimulated with *E. coli* lipopolysaccharide for 24 h, both with and without a 30 min pretreatment with 5 µM or 10 µM of curcumin. Other wells were treated with 10 µM of the p38 MAPK inhibitor (SB203580) or 10 µM of the NF-kB inhibitor (Bay 11-7082). Control wells were treated with the same volume of the DMSO vehicle used to dilute these compounds. These culture supernatants were aliquoted and stored at -80 C. These samples were thawed only once and used in ELISA assays to quantify the secreted PGE2, IL-6 and TNF-α. These assays were performed according to the manufacturer's instructions (R&D Systems) and the target protein concentration was normalized to the total protein content determined by the Lowry method (DC assay, Bio-Rad).

Activation of signaling pathways (Western-blot)

For these experiments, 3 x 10^5 cells were grown for 24 h in each well of six-well plates, routinely de-induced for 8 h and subsequently treated with 5 or 10 µM of curcumin 30 minutes before the stimulation with bacterial LPS. Control experiments were performed with the addition of 10 µM of SB203580, 10 µM of Bay 11-7082 or of the same volume of DMSO vehicle. LPS stimulation was maintained for 10, 30 and 60 minutes in the short-term experiments, or for 12, 24 and 48 hours. At the end of each experimental

period, whole-cell lysates were harvested by scraping the cells in sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by sonication for 10 s and heat-denaturation at 95^{o}C for 5 min. To experiments evaluating the nuclear and cytosolic protein expression,

Total protein content was quantified by Lowry method (DC Assay, Bio-Rad). For western blotting, 30 µg of total protein were separated on 10% Tris-HCl polyacrylamide gels run at 100 V for 60 min and subsequently electrotransferred to nitrocellulose membranes for another 60 min in a semidry apparatus at 110 mA/gel. The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 h at room temperature and then probed with the primary antibodies overnight at 4^{o}C. The presence of the primary antibodies was detected by using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA). Digital images of the radiographic films exposed to the membranes membranes were obtained on a gel documentation system (GelDoc XT; Bio-Rad). Expression levels of beta-actin were determined to verify equal loading of proteins.

Results

Cytotoxicity of curcumin

Initially, we performed a dose-response evaluation of the cytotoxic effects of curcumin using a selective NF-kB inhibitor (Bay 11-7082) as a positive control. Since the effects of curcumin may be related to its cytotoxic effects, to avoid misinterpretation of the information collected on the effects of curcumin on gene expression and activation of

signaling pathways, we intended to use the highest non-cytotoxic concentration of both curcumin and Bay 11-7082. RAW 264.7 cells were treated with the indicated concentrations of curcumin (5 μM - 100 μM) and Bay (10 μM - 40 μM) for 24 hours. The results of the MTS-based assay of untreated control cells were set to 100% and the results for cells treated with DMSO vehicle, curcumin and Bay 11-0782 are presented as relative increase or decrease to untreated control cells. Concentrations of curcumin up to 10 μM produced no change in cell viability, whereas higher concentrations of curcumin ($\geq$ 25 μM) caused significant citotoxicity (Figure 1). Concentrations of Bay 11-7082 above 40 μM produced a decrease of 90% in cell viability compared to untreated control cells. We subsequently used 10 μM of curcumin and Bay 11-7082 as the maximum concentrations for the subsequent experiments. Cytotoxicity of SB203580 was not assessed because we had data from other experiments (not shown) indicating that 10 μM of SB203580 caused no change in cell viability.

Curcumin inhibits LPS-induced cytokine expression

LPS stimulation induced a significant increase on the expression of Ptps2 (the murine homolog to human cyclooxygenase-2) mRNA by the macrophages. This increase was inhibited by pre-treatment of the cells with curcumin in a concentration-dependent manner; but a significant decrease was only observed with 10 μM of curcumin. In the absence of LPS stimulation, curcumin and the biochemical inhibitors (SB203580 and Bay 11-7082) induced a slight increase of PGE2-synthase mRNA. Interestingly, in the presence of LPS stimulation, the NF-kB inhibitor increased the LPS-induced expression of Ptps2 mRNA (Figure 2). Detectable levels of PGE2 protein were only observed in the samples from LPS-treated cells, indicating that curcumin (at 5 and 10 μM) and both SB203580 and Bay 11-7082 completely abrogated LPS-induced PGE2 protein expression by macrophages. (Figure 3).

Curcumin, but not the biochemical inhibitors, induced TNF-α and IL-6 mRNA expression in the absence of LPS stimulation. However, in cells stimulated with LPS curcumin dose-dependently inhibited LPS-induced expression of IL-6, whereas inhibition of TNF-α mRNA was observed only with 10 μM of curcumin. Neither SB203580 nor Bay 11-7082 inhibited LPS-induced IL-6 and TNF-α mRNA (Figure 2). IL-6 and TNF-α protein were undetectable in the absence of LPS stimulation; and pre-treatment of the cells with 10 μM of curcumin significantly inhibited LPS-induced secretion of IL-6 and TNF-α, which were only slightly affected by the p38 MAPK inhibitor, SB203580 (Figure 3).

Curcumin inhibits LPS-induced activation of NF-kB, p38 and ERK MAPKinases

Since NF-kB is considered a prime target of curcumin and also based on the relevance of both NF-kB and MAPKinase signaling pathways for LPS-induced cytokine gene expression, we next evaluated the modulation of these pathways by curcumin. Short-term stimulation experiments were performed and the biochemical inhibitors of p38 MAPK (SB203580) and NF-kB (Bay 11-7082) were used for comparative purposes. In the negative controls, equivalent volumes of the DMSO vehicle replaced curcumin and the biochemical inhibitors. Macrophages were treated with curcumin (5 μM and 10 μM) for 30 minutes followed by stimulation with LPS (1 μg/mL) for 10, 30 and 60 minutes. LPS induced activation of NF-kB, p38 and ERK within 10 minutes and JNK was activated 30 minutes after the stimulation. This activation was sustained, in all signaling pathways for a least 60 minutes. Pre-treatment of the cells with curcumin inhibited LPS-induced activation of all three MAPKinases as well as of NF-kB in a concentration-dependent manner 30 minutes after stimulation. Biochemical inhibitors were more effective also 30 minutes after stimulation, but there was a lack of specificity, with the

NF-kB inhibitor (Bay 11-7082) affecting activation of JNK and ERK MAPKinases besides p38 MAPK (Figure 4).

Curcumin increases the socs-1 and socs-3 activation in macrophages stimulated with LPS

To evaluate the role of curcumin on activation of socs-1 and socs-3, macrophages were treated with curcumin (5 μM and 10 μM) for 30 minutes followed by stimulation with LPS (1 μg/mL) for 12, 24 and 48 hours. Curcumin increased the socs-1 expression 12 hours and 48 hours after the LPS stimulation. Regarding to socs-3, LPS simulation inhibited its activation in all periods analyze, but the treatment with curcumin restaured the socs-3 levels 12 (5um) and 24 hours (10um) after the stimuli.

Discussion

In this study, we show that curcumin is a potent inhibitor of the expression of PGE2, IL-6 and TNF-α by LPS-stimulated macrophages. Macrophages are major sources of inflammatory cytokines and pro-inflammatory radicals, participating in both the innate and adaptive immune responses. These inflammatory mediators can be both produced by macrophages with paracrine effects and utilized by them in autocrine-regulatory fashion. When activated by an external stimulus these cells migrate to the affected site and are able to internalize and digest bacteria and other cells. Besides, macrophages can scavenge toxic compounds produced by the metabolism and to secrete soluble inflammatory mediators that can kill bacteria, parasites, and viruses and contribute to the activation of other cell types and to the walling off of parasites (Auffray *et al.*, 2009).

In spite of this protective nature, activation of the immune system and the host-derived cytokines causes host tissue degradation as collateral damage. Thus, modulation of the

host response by reducing the production of inflammatory cytokines is an actively-pursuit therapeutic strategy; and due to their relevance on the immune responses, macrophages are a prime target. In general, our results agree with those of other studies that reported an anti-inflammatory effect of curcumin.

The effect of curcumin on the induction of NO syntase in RAW264.7 cells activated by LPS (1 μg/ml) and IFN-γ (100 U/ml) was investigated and compared with various non-steroidal anti-inflammatory drugs (aspirin, piroxicam, indomethacin, ibuprofen, sulindac, sodium salicylate) (Brouet *et al.*, 1995). All the anti-inflammatory agents, except sulindac, inhibited NO production in a concentration-dependent manner; however the induction of NOS was inhibited by curcumin at the lowest concentration among the compounds tested. Maximum inhibition was observed when curcumin was added to the culture medium at the time of activation of macrophages with LPS and IFN-γ. When curcumin was added 6 or 8 h after activation or 2h before activation, the inhibition was less effective. Since we intended to evaluate the effects of curcumin from a cell signaling perspective, we added curcumin to the cultures 30 min before the stimulation, which is usually done with biochemical inhibitors signaling. As opposed to the reciprocal regulation of regulation of NOS at mRNA and protein levels, our results demonstrate a lack of transcription-translation coupling. Considering the fact that RNA and protein samples were harvested simultaneously from the same wells, this may suggest the influence of post-transcriptional regulation, which has been shown to play a role for the target genes we evaluated (TNF-α and IL-6) (Basler *et al.*; Neininger *et al.*, 2002; Zhao *et al.*, 2008). Alternatively, the lack of correspondence between mRNA and protein levels may merely reflect the lag between the regulation of mRNA transcription and protein synthesis and secretion. The mechanism of regulation of cytokine expression by curcumin will be explored in future studies.

Curcumin was recently shown to dose-dependently inhibit the expression of TNF-α and IL-1β by LPS-stimulated RAW264.7 cells (Chen *et al.*, 2008). In this study, cells were treated with similar (5µm, 10µm) and higher (20µm, 30µm) concentrations of curcumin in comparison to the present report. The authors (Chen *et al.*, 2008) used a 2 h pre-treatment with curcumin before stimulating with LPS. The nature of LPS used was also different from the LPS used in the present study: LPS from *E. coli* we used is a known activator of TLR4; whereas LPS from the periodontopathogenic microorganism *Porphyromonas gingivalis* used in the study by Chen et al. (2008)(Chen *et al.*, 2008) activates preferentially TLR2. These differences on the curcumin treatment and source of LPS used can account for the weak inhibition of both TNF-α and IL-1β with lower concentrations of curcumin reported by Chen et al. (2008) (Chen *et al.*, 2008). In our study, concentrations above 20 µm of curcumin showed be toxic for this cell line; moreover even lower concentrations of curcumin (5 µm) markedly decreased TNF-α protein production by RAW 264.7 cells stimulated with TLR4-activating LPS. The concentrations of curcumin used in the present study were similar to those of Abe et al. (Abe *et al.*, 1999) who showed a marked inhibition of IL-1β, TNF-α, IL-8 and MIP-1α in LPS-stimulated human monocytes and alveolar macrophages; and much lower than the maximum of 1 µM used by Jain et al. (Jain *et al.*, 2009) who reported a significant but less potent inhibition of IL-6 and TNF-α in human monocytes cultured in high glucose.

We used bacterial LPS as an external stimulus to study the effect of curcumin on innate immune signaling by Toll-like receptor 4 (TLR4). TLR activation recruits specific adaptor molecules, such as MyD88, which then bind the interleukin-1 receptor-associated kinase (IRAK) in the cytoplasmic tail of the receptor. The signal is thereafter transmitted through a chain of signaling molecules involving the tumor necrosis factor

receptor-associated factor-6 (TRAF6), a common upstream activator of MAPKinases and NF-kB. TLR4 activation also leads to a delayed activation of NF-kB in a MyD88-independent manner. This delayed activation of NF-kB is independent of the rapid activation mediated by the MyD88-dependent pathway. Activation of NF-kB and MAPKinases ultimately leads to increased expression of genes involved in the activation of innate immunity, mainly pro-inflammatory cytokines (Akira *et al.*, 2003; An *et al.*, 2002; Anderson, 2000; Kirkwood *et al.*, 2009).

Curcumin has been shown to inhibit various signal transduction pathways such as NF-kB, AP-1, MAPKinases and protein kinase C (Huang *et al.*, 1991; Rafiee *et al.*, 2009). In human intestinal epithelial cell line HT29 stimulated with TNF-α and IL-1β, curcumin was able to inhibit NF-kB and MAPKinases, such as p38 and JNK, but did not inhibit activation of ERK1/2 (Moon *et al.*, 2006). On the other hand, inhibition of all three MAPKinases was also observed in human esophageal cells stimulated by low pH (Rafiee *et al.*, 2009) as well as in an adipocyte cell line treated with palmitate (Wang *et al.*, 2009) and in human microvascular endothelial cells stimulated with VEGF (Binion *et al.*, 2008). Inhibition of NF-kB activation by curcumin in RANKL- and LPS-stimulated murine macrophages reduces osteoclastogenesis and IL-12 production (Bharti *et al.*, 2004; Kang *et al.*, 1999). Interestingly, curcumin markedly inhibits LPS-induced production of IL-12, but not of IL-10, by macrophages and affects the adaptive immune response by inhibiting Th1 cytokine profile (Kang *et al.*, 1999). Suppression of NF-κB activation by curcumin inhibited the expression of pro-inflammatory cytokines in human articular chondrocytes stimulated with TNF-α and IL-1β (Shakibaei *et al.*, 2007). This information indicates the therapeutic potential of curcumin in conditions associated with chronic inflammation and bone resorption.

We have observed inhibition of NF-kB and of all three MAPKinases by curcumin in macrophages, suggesting that its potent inhibitory effect on LPS-induced cytokine expression may be related with the modulation of these signaling pathways. The fact that curcumin inhibits all these pathways simultaneously suggests that it may be acting on an upstream activator or even on the extracellular portion of TLR4, by affecting both ligand-induced and ligand-independent dimerization (Youn *et al.*, 2006). Importantly, the marked effect of curcumin on cytokine expression may be dependent on this simultaneous inhibition of multiple signaling pathways, as indicated by the comparison with NF-kB and p38 MAPK inhibitors. It will be interesting to define the specific target(s) of curcumin in the TLR4 signaling pathway. This prominent role of curcumin on the activation of innate immunity may have potential to be explored in the modulation of host response in chronic conditions associated with infectious stimuli, such as periodontal diseases and lower digestive tract diseases.

In summary, we have shown that curcumin modulates TLR signaling in macrophages and effectively inhibits cytokine gene expression induced by TLR stimulation indicating its therapeutic potential as a host modulatory agent in chronic infectious conditions.

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Figure Legends

Figure 1 – Cytotoxicity of curcumin and NF-kB inhibitor (Bay 11-0782) to Raw 264.7 cells. Raw macriophages were grown in 96-well plates, de-induced in medium containing 0.3% FBS and treated with the indicated concentrations of curcumin (5 -100 μM) or Bay 11-0782 (10 - 40 μM) for 24 hours. Cell viability was determined by an MTS-based assay (Left) 1 hour and (Right) 2 hours after the addition of MTS. The average value of the absorbance reading of DMSO-vehicle treated cells was set to 100% and the results for cells treated with curcumin and Bay 11-0782 are presented as relative increase or decrease. Bars represent means and vertical lines standard deviations of 3 independent experiments.

Figure 2 - Curcumin inhibits LPS-induced pro-inflammatory cytokine expression at the mRNA level. Raw 264.7 cells were de-induced in 0.3% FBS, treated with 10 μM of NF-kB (Bay 11-0782) or 10 μM of p38 MAPK (SB203580) inhibitors, curcumin (5 or 10 μM) or the same volume of DMSO vehicle for 30 min and then stimulated with *E.coli* LPS (1 ug/mL) or with the same volume of PBS vehicle (control) for 24 h. Total RNA was harvested and used for RT-qPCR performed with pre-designed primers and probes for the indicated target genes and to the housekeeping GAPDH using TaqMan reagents. The results were analyzed by the delta-Ct method and expression of target genes was normalized to beta-actin expression. Data is presented as fold change to DMSO-vehicle treated and unstimulated (control) cells. (*) indicates significant increase, $p < 0.05$, in comparison to control, (+) indicates significant decrease, $p < 0.05$, in comparison to LPS. Bars indicate means and vertical lines standard error of mean of three independent experiments analyzed in duplicate.

Figure 3 – Effective inhibition of inhibits LPS-induced pro-inflammatory cytokine expression at protein level by curcumin. Raw 264.7 cells were de-induced in 0.3% FBS,

treated with 10 μM of NF-kB (Bay 11-0782) or 10 μM of p38 MAPK (SB203580) inhibitors, curcumin (5 or 10 μM) or the same volume of DMSO vehicle for 30 min and then stimulated with *E.coli* LPS (1 ug/mL) or with the same volume of PBS vehicle (control) for 24 h. Cell culture supernatants were harvested, aliquoted and used in ELISA tests to quantify the expression of the target cytokines. These tests were performed according to the manufacturer's instructions and the results for each sample were normalized to the concentration of total proteins determined by a Lowry-based microassay (DC assay, Bio-Rad). (*) indicates significant difference, $p< 0.05$, in comparison to control, (+) indicates significant difference, $p< 0.05$, in comparison to LPS. Bars indicate means and vertical lines standard error of mean of three independent experiments analyzed in duplicate.

Figure 4 – Inhibition of LPS-induced activation of NF-kB and all three MAPKinases. Raw 264.7 cells were de-induced in 0.3% FBS, treated with 10 μM of NF-kB (Bay 11-0782) or 10 μM of p38 MAPK (SB203580) inhibitors, curcumin (5 or 10 μM) or the same volume of DMSO vehicle for 30 min and then stimulated with *E.coli* LPS (1 ug/mL) or with the same volume of PBS vehicle (control) for 10, 30 and 60 min. Cell lysates were harvested at the indicated time points in SDS-containing sample buffer supplemented with protease and phosphatase inhibitor cocktails (Complete and Phos-Stop, Roche). Total concentration of protein was determined with a Lowry-based microassay (DC assay, Bio-Rad) and 30 μg of each sample were diluted in LDS-containing running buffer supplemented with DTT, heat-denatured, electrophoresed in 10% Tris-Acrilamide gels and transferred to 0.2 μM nitrocellulose membranes in a semi-dry transfer apparatus. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65 (NF-kB), p38, JNK and ERK MAPKinases. Expression levels of constitutive housekeeping beta-actin are shown to

confirm equal protein loading. The images are representative of three independent experiments.

Figure 1

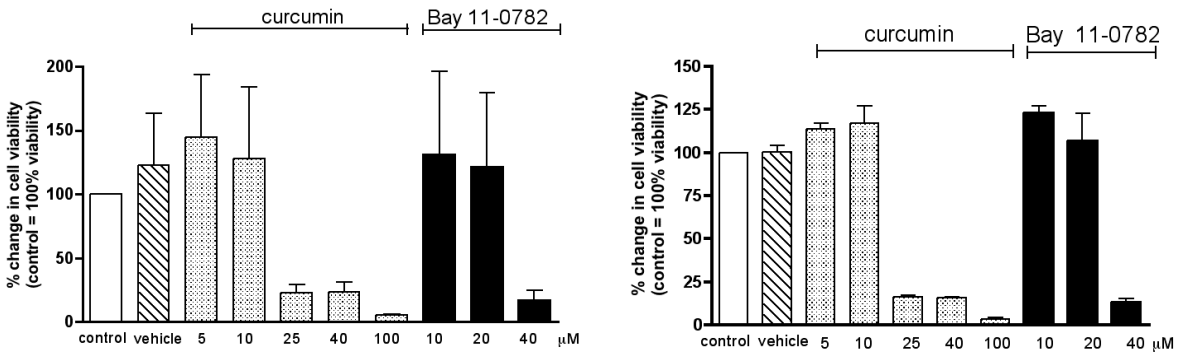
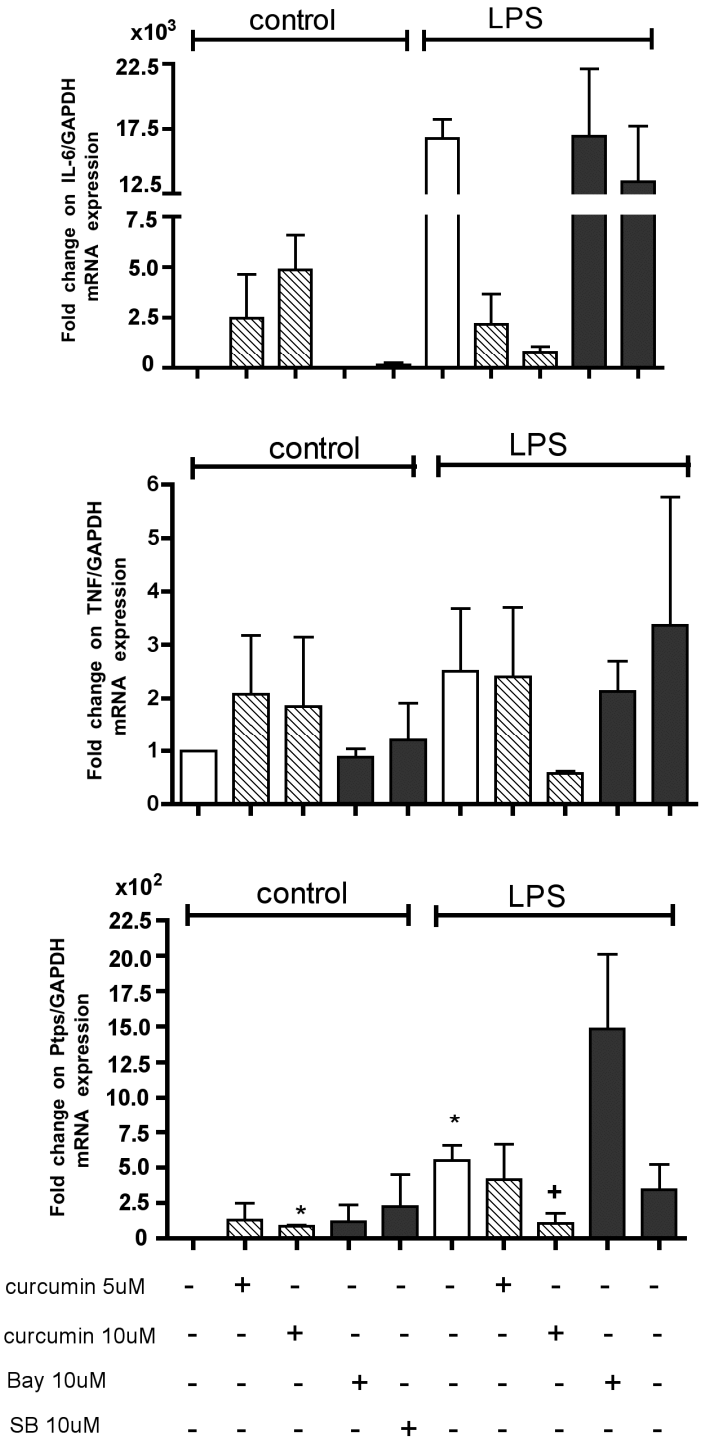


Figure 2


control
LPS
x10^3
22.5
17.5
12.5
7.5
5.0
2.5
0
Fold change on IL-6/GAPDH mRNA expression
control
LPS
6
5
4
3
2
1
0
Fold change on TNF/GAPDH mRNA expression
x10^2
control
LPS
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0
*
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Fold change on Ptps/GAPDH mRNA expression
curcumin 5uM - + - - - - + - - -
curcumin 10uM - - + - - - - + - -
Bay 10uM - - - + - - - - + -
SB 10uM - - - - + - - - - -

Figure 3

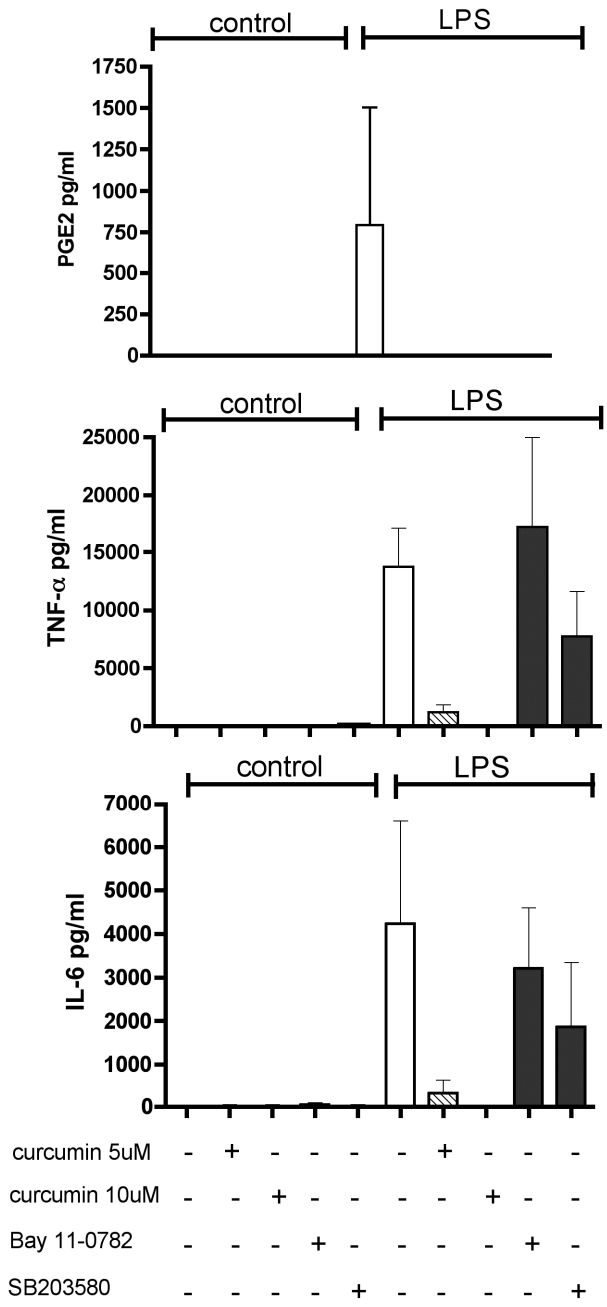
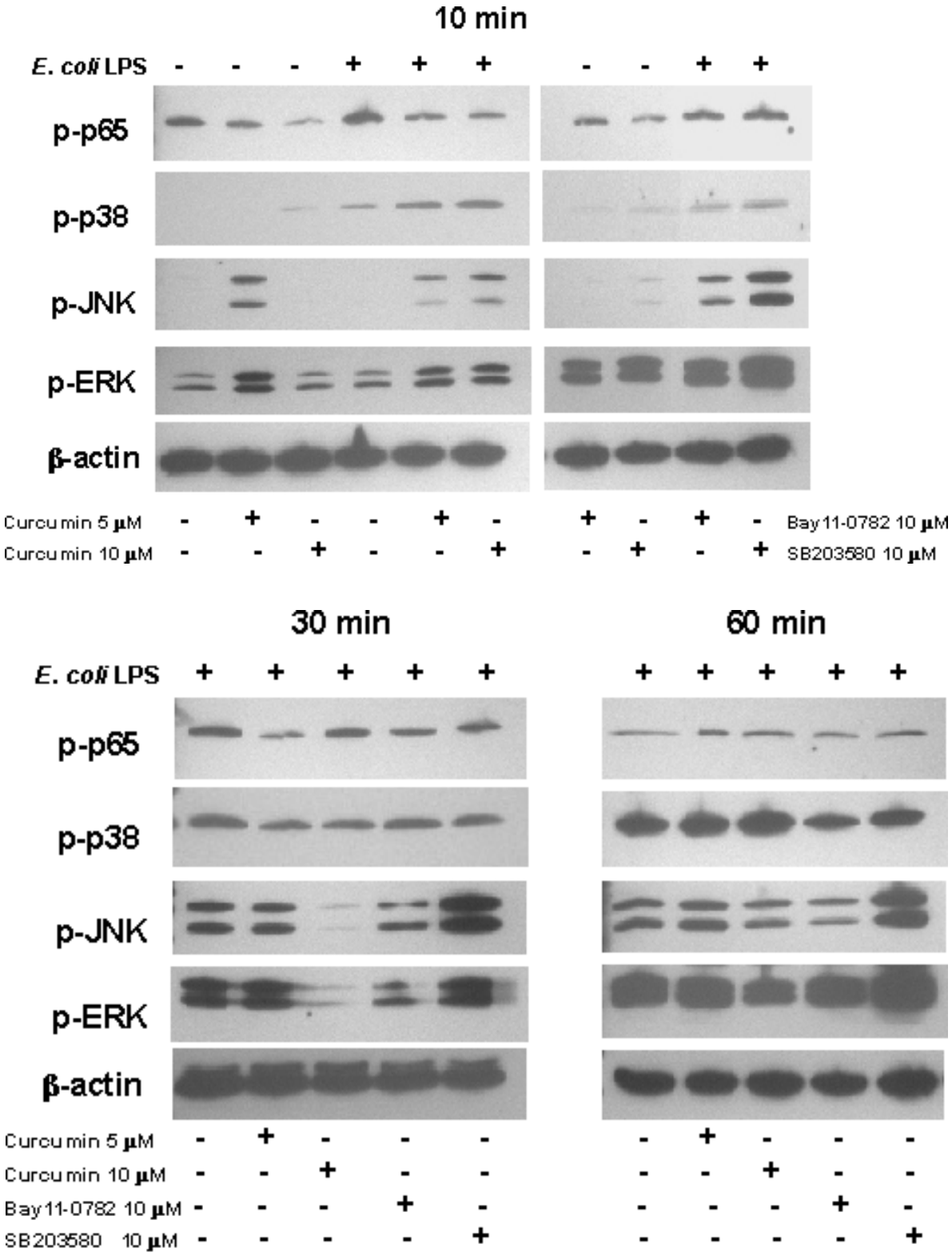

control
LPS
PGE2 pg/ml
1750
1500
1250
1000
750
500
250
0
control
LPS
TNF-α pg/ml
25000
20000
15000
10000
5000
0
control
LPS
IL-6 pg/ml
7000
6000
5000
4000
3000
2000
1000
0
curcumin 5uM - + - - - - + - - -
curcumin 10uM - - + - - - - + - -
Bay 11-0782 - - - + - - - - + -
SB203580 - - - - + - - - - +

Figure 4


10 min
E. coli LPS
p-p65
p-p38
p-JNK
p-ERK
β-actin
Curcumin 5 μM
Curcumin 10 μM
Bay11-0782 10 μM
SB203580 10 μM
30 min
60 min

CAPÍTULO 2

Potent anti-inflammatory effects of systemically-administered curcumin modulates periodontal disease in vivo

Running title: Curcumin inhibits periodontal disease in vivo

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Key-words: Curcumin, inflammation, periodontal disease, NF-kB, p38 MAPK

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Abstract

Background: Curcumin is a plant-derived dietary spice with various biological activities, including anti-tumoral and anti-inflammatory. Its therapeutic applications have been studied in a variety of conditions, such as rheumatoid arthritis, colon cancer and depression; but no studies evaluated the effects of curcumin on periodontal disease in vivo.

Methods: Experimental periodontal disease was induced in rats by placing cotton ligatures around both lower first molars. Curcumin was given to the rats by daily oral gavage in two doses (30 and 100 mg/Kg) during the whole 2 weeks of the experiment. Control animals received ligatures but only the corn oil vehicle by gavage and negative control animals were untouched. RT-qPCR and ELISA were used to determine the expression of IL-6, TNF-alpha and PGE-2 synthase on the gingival tissues. The inflammatory status was evaluated by stereometric analysis, whereas modulation of p38 MAPK and NK-kB signaling was assessed by western blot.

Results: Curcumin effectively inhibited cytokine gene expression at mRNA and protein levels and dose-dependently inhibited activation of NF-kB in the gingival tissues. p38 MAPK activation was not inhibited by curcumin. Curcumin-treated animals also presented a marked reduction on the inflammatory cell infiltrate and increased collagen content and fibroblastic cell numbers.

Conclusions: Curcumin did not induce detectable adverse effects, and its potent anti-inflammatory effect suggests it may have a therapeutic potential in periodontal diseases.

Introduction

Phytochemicals are naturally occurring substances found in plants. There has been considerable public and scientific interest in the use of phytochemicals derived from dietary components to combat human diseases, especially the two major causes of death in the developed world: cardiovascular diseases and cancer [1]. Curcumin (diferuloymethane), is one such phytochemical, which is a major constituent of the yellow spice turmeric derived from the rhizomes of Curcuma spp. [2].

Curcumin has potent anti-inflammatory, anti-carcinogenic and antioxidant activities, and a number of pre-clinical trials have been conducted to assess its therapeutic potential [3-5]. Following oral administration, curcumin has been shown to prevent cancer in the colon, skin, stomach, liver, lung, duodenum, soft palate and breasts of rodents [6, 7]. In a rat model of acute ulcers, curcumin potently attenuates the ulcer activity by preventing glutathione depletion, lipid peroxidation, and protein oxidation. Both, oral and intraperitoneal administration of curcumin blocked gastric ulceration in a dose-dependent manner [8]. In animal studies of arthritis, oral administration of curcumin decreased the levels of inflammatory glyprotein, Gp A72, with a reduction in inflammatory response in the paws [9].

The anti-inflammatory properties of curcumin seem are mediated by the modulating the activity of signaling pathways and transcription factors, especially NF-KB, AP-1 and MAPKinases [10]. Down regulation of the activation of NF-kB and MAPKinases by curcumin suppresses the expression of IL-6, IL-1β, TNF-α, MMP-2 and MMP-9 in the late phase of experimental acute pancreatitis [11], in the modulation of arthritis [12-15] in the prevention and healing of indomethacin-induced gastric ulcer [8], in the treatment of inflammatory bowel disease and Crohn`s disease [16-18].

Periodontal disease initiation and progression occurs as a consequence of the host response to microorganisms of the dental biofilm. Besides their role as stimulants of the host response, periodontal pathogens release harmful by-products and enzymes that break down extracellular matrix components, such as collagen, as well as host cell membranes. Once the host response is initiated, various inflammatory molecules, such as cytokines and prostaglandins are released from leukocytes, fibroblasts or other host tissue-derived cells [19-21]. Chronic periodontal inflammation perpetuates and amplifies itself through numerous autocrine and paracrine regulatory loops of the inflammatory mediators, acting on cells within the periodontal microenvironment. An

improper immune response leads to overproduction of inflammatory cytokines and consequently periodontal attachment loss and bone resorption. Host modulation therapeutic strategies aimed at inhibition of the progression of inflammatory bone loss associated with periodontitis include the blockage of inflammatory cytokines. Recently, the cell signaling pathways that regulate the expression of inflammatory mediators have become promising therapeutic targets [19].

Although a range of biological and pharmacological activities of curcumin have been reported, its therapeutic potential for destructive periodontal disease is poorly understood. To the best of our knowledge, this is the first study evaluating the effect of curcumin on the modulation of periodontal disease in vivo.

Besides its anti-inflammatory properties, curcumin was also shown to improve wound healing by increasing collagen deposition, angiogenesis and the density of fibroblasts, reducing the radiation-induced delay in wound repair [22]. Interestingly, curcumin-treated wounds presented not only a greater number of fibroblasts but also more infiltrating macrophages and neutrophils compared to untreated wounds [23, 24]. These studies demonstrated that treatment with curcumin resulted in faster closure of wound, better regulation of granulation tissue formation and induction of growth factors [24], all features that can be extremely useful in the setting of periodontal disease.

These findings prompted us to investigate the effect of systemically administered curcumin on the inflammatory response during the course of ligature-induced periodontal disease in rats. The present study was undertaken to determine whether curcumin could inhibit connective tissue breakdown in ligature-induced periodontitis in rats. To obtain greater insight into the anti-inflammatory effects of curcumin, we assessed the modulation of signaling pathways (p38 MAPK and NF-kB) and the expression of IL-6, COX-2 and TNF-α in the periodontal tissues.

Materials and methods

Experimental design

All the experimental protocols were approved by the Ethical Committee for Animal Experimentation (CEEA) of the School of Dentistry at Araraquara – UNESP and performed in accordance with the guidelines from the Brazilian College for Animal Experimentation (COBEA).

Forty male Holtzman rats (Rattus norvegicus albinus Holtzman), weighing between 100 and 200 g, were randomly distributed into four experimental groups comprising 10 animals each. The rats were kept in a room with controlled temperature (21 ± 1^{o}C) and

humidity (65–70%) and a 12 h–12 h light–dark cycle. Animals were fed standard rat chow and water *ad libitum*. To induce periodontitis, three groups of rats were anesthetized by intramuscular administration of ketamine (Francotar, Virbac of Brazil Ind. and Com. Ltd, São Paulo, Brazil; 80 mg/kg body weight) and xylazine (Virbaxil, Virbac of Brazil Ind. And Com. Ltd, São Paulo, Brazil; 20 mg/kg body weight), and cotton threads were tied around the first molars bilaterally.

Administration of curcumin started the day before the placement of ligatures for the induction of periodontal disease. Curcumin was administered daily by oral gavage in two different doses: one group received the lower dose of 30 mg/Kg/ body weight, and the other group received 100 mg/Kg/body/weight. Control animals were given the same volume of the corn oil vehicle. Fifteen days after the placement of ligatures the animals were sacrificed and the mandibular jaws were hemisected. Some of the block sections including 1^{st} and 2^{nd} molars with their surrounding tissues were submitted to routine histological processing for descriptive and stereomteric evaluation, whereas other blocks were used for the extraction of total RNA and protein. For stereometry, 3 blocks including the lower molars of the control group and 4 blocks of the lower molars of each experimental group were analyzed.

These tissue blocks were immersed directly in 10% buffered formalin fixative solution for 48 h and decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) during 2-3 months, under agitation at room temperature. Each specimen consisted of a section containing the 1st and 2nd molars and their surrounding alveolar process and was included in paraffin blocks. Serial 4 μM sections were obtained in the bucco-lingual direction and stained with hematoxylin-eosin (HE).

Stereometry

The analysis was conducted by a single examiner that was blind to the experimental groups using an optical microscope (Diastar Cambridge) set at 200 X magnification. Semi-serial sections of 4 μM were obtained from the tissue blocks on a buccal-lingual orientation. A total of 3 sections, spaced 100 μM from each other, were evaluated per tooth. A 50 x 50 μM grid was overlayed the histological images allowing the analysis of an 'area of interest' of 2,500 μM^2. Two grids composed of 5 x 5 10 μM squares were used in each histological image: one was positioned with its lower border 25 μM below the top of the alveolar bone crest and perpendicularly to the root surface, and the other was positioned with its upper border at the base of the junctional epithelium,

representing the ‘bone crest’ and ‘submarginal’ areas, respectively. In both cases, the lateral border of the grids was always positioned over the most prominent part of the root surface in the area. Each one of the 25 points of the grid projected on each section was counted and the proportion of collagen, fibroblastic cells and inflammatory cells (distinguished by the morphological characteristics) in the area of interest was determined as percentage of the total points counted. A total of three images obtained from equally spaced slides (spanning 900 uM of the buccal-lingual aspect of the molars) were evaluated from each animal. Slides from at least three different animals in each experimental group were used.

Evaluation of cytokine gene expression at the mRNA level (RT-qPCR)

Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen Corp) according to the manufacturer’s instructions. Concentration of RNA was determined by spectrophotometry and 700 ng of total RNA were reverse-transcribed into cDNA using random hexamers as primers (High Capacity cDNA synthesis kit, Applied Biosystems). The relative abundance of the transcripts of the candidate inflammatory genes were measured by real-time reverse transcription-PCR (RT-PCR) using Taqman chemistry and pre-designed sets of primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) on a StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate on a final reaction volume of 30 µL that included Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) for each target gene: TNF-α (tumor necrosis factor alpha), NM_013693; IL-6 (interleukin 6), NM031168; Ptgs-2 (prostaglandin-endoperoxide synthase-2), NM_011198; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), NM_008084; and cDNA template (corresponding to 30 ng of cDNA). Optimized thermal cycling conditions were: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 s and 60^{o}C for 1 min. For each sample, analyses of gene expression were performed in duplicate. The experiments were with samples isolated from at least three different animals in each experimental group. To normalize the amount of mRNA present in each reaction, the expression of GAPDH, which was not altered by the experimental conditions, was used as a housekeeping gene. To compare the expression levels among different samples, the relative expression level of the genes was calculated using the comparative ΔCT method using the thermocycler’s software.

Activation of signaling pathways (Western-blot) and determination of cytokine gene expression at the protein level (ELISA)

Total proteins were extracted from gingival tissue samples using a detergent-based extraction buffer (T-PER, Tissue Protein Extraction Reagent – Pierce Biotechnology) containing a protease inhibitor cocktail (Protein Stabilizing Cocktail – Santa Cruz Biotechnology) according to manufacturer's instructions (Pierce Biotechnology). The tissue samples were macerated in the buffer (50 μL / mg of tissue) and centrifuged for 5 min at 13,000 RPM at 4°C. The proteins were quantified using Lowry method (DC assay, Bio-Rad Laboratories) and 40 μg of total protein were added to a SDS sample buffer containing 2% SDS, DTT as a reducing agent, glycerol and bromophenol blue dye (Cell Signaling), heated-denaturated at 97° C for 5 min and chilled on ice of 5 min before loading on 10% SDS-polyacrylamide gels.

Electrophoresis on discontinuous acrylamide gels were carried out at 100 V for 90 min and subsequently electro-transferred to 0.2 μM nitrocellulose membranes using 300 mA constant current for 1 hour. The membranes were blocked for 1h in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 and subsequently washed for 5 min (3 times) with TBS-0.1% Tween at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C (1:100 dilution in PBS – phospho-p65 and phospho-p38 – Cell signaling). Membranes were washed in TBS-T buffer for 5 minutes (3 times) and incubated with secondary antibodies conjugated to horseradish peroxidase (1:1000 dilution in the blocking buffer) for 1 hour at room temperature, washed again with TBS-T buffer (5 min, 3 times). Detection of bands was carried out on radiographic film by using a chemiluminescence system (Lumi-Glo, Cell Signaling).

Proteins from these same samples were also used to in the ELISA assays to determine the concentration of PGE_2, IL-6 and TNF-α. These assays were performed according to the manufacturer's instructions (R&D Systems) and the results were normalized to the total concentration of protein in the samples. Samples from at least three different animals in each experimental group were used and assayed in duplicate.

Data analysis

The purpose of data analysis was to compare the results from curcumin-treated animals with those of vehicle treated control animals. Considering the two experimental groups treated with different doses of curcumin (30 and 100 mg/kg body weight) as independent variables, we used non-paired t-tests for the comparisons between all the groups (control x 30 mg/Kg of curcumin, control x 100 mg/Kg of curcumin and 30 x 100 mg/Kg of curcumin) with a significance level of 5%.

Results

Curcumin did not affect body weight or the behavior of the experimental animals in comparison to vehicle-treated controls. No animal died during the experimental period, indicating the lack of severe adverse effects associated with curcumin administration.

Curcumin completely abrogates PGE2-synthase (murine Cox-2 analog) mRNA expression in ligature-induced periodontal disease

To investigate the effect of curcumin on ligature-induced inflammatory gene expression the total RNA and protein were extracted from gingival tissues surrounding the lower first molars of rats with or without ligature placement. These samples were used for RT-qPCR and ELISA experiments, respectively. Interestingly, animals treated with the higher dose of curcumin (100 mg/Kg) presented a marked increase on PGE2-s mRNA levels in comparison to control animals. Ligature placement increased PGE2-s mRNA in the tissues, but treatment with curcumin completely inhibited this increase (Fig. 1). We could not detect PGE2 expression at the protein level in our samples, probably due to the reduced level of expression.

Curcumin effectively inhibits IL-6 and TNF-α gene expression

At protein level, the treatment with 100 mg/Kg of curcumin induced IL-6 protein expression in non-diseased periodontal tissues; however, there was a dose-dependently and significant inhibition of IL-6 mRNA (Fig. 2) and protein (Fig. 3) expression in periodontally-diseased tissues of animals treated with both doses of curcumin. Interestingly, greater inhibition of IL-6 was associated with the lower dose (30 mg/Kg) of curcumin. TNF-alpha mRNA expression in ligature-induced periodontal disease was completely inhibited by curcumin (Fig. 4). Both doses of curcumin inhibited TNFα protein expression in periodontal tissues surrounding ligated teeth; however similarly to the regulation of IL-6 this inhibition reached statistical significance only for the 30 mg/Kg dose (Fig 5).

Curcumin inhibits ligature-induced activation of NF-kB, but not of p38

Since NF-kB is considered a prime target of curcumin and also based on the relevance of both NF-kB and MAPKinase signaling pathways for inflammatory cytokine expression, we next evaluated the modulation of these pathways in periodontally diseased tissues by orally administered curcumin.

The placement of ligature elicited the inflammatory process and stimulated activation of NF-κB. Administration of curcumin produced a marked, dose-dependent inhibition of

NF-kB activation in periodontally-diseased tissues. Surprisingly, activation of p38 MAPK was reduced in periodontally-diseased tissues. The lower dose of curcumin (30 mg/Kg) did not change the reduced activation status of p38 MAPK associated with ligature-induced periodontal disease, whereas the higher dose (100 mg/Kg) restored the activation status observed in healthy periodontal tissues. (Fig 6).

Curcumin inhibits the ligature-induced inflammatory process and enhances the deposition of collagen:

A stereometric analysis was performed to correlate the information on the effects of curcumin on the activation of signaling pathways and on the expression of pro-inflammatory cytokines with the overall modulation of the inflammatory status in the periodontally diseased tissues. Ligature-induced periodontitis induced a clear and obvious inflammatory process, characterized by increased cell and vascular densities and reduced collagen content. Administration of curcumin had a suppressive effect on the inflammation, as evidenced by the absence of inflammatory cells in the submarginal and bone crest areas (Fig 7). Collagen content in the tissues was also increased in curcumin-treated animals, which may be due to the inhibition of inflammation and, consequently, of the collagen degradation associated (Fig 8). These results indicate a clear correlation between the histological inflammation status and the expression of pro-inflammatory mediators.

Discussion

In this report, we investigated the anti-inflammatory effect of curcumin in ligature-induced periodontitis in rats. The current study, for the first time, demonstrated that orally administered curcumin effectively reduces inflammation and connective tissue breakdown in this experimental periodontitis model. Moreover, we provide evidence that this effect might be explained, at least in part, by the inhibition of IL-6, PGE-2 and TNF-α expression, as a result of the modulation of NF-κB activation.

The relevance of these cytokines is supported by studies showing that these cytokines have all been found to be significantly elevated in diseased periodontal sites compared with healthy or inactive sites [25-31] and have been positively correlated with increased probing depth and attachment loss [19, 28, 30].

Recent studies have found that curcumin have the ability of suppress NF-κB [1, 32-35] and MAPKinases [1, 32, 35] thus reducing the production of inflammatory cytokines such as IL-6 [11, 32, 34, 36], TNF-α [11, 32, 36, 37] and PGE-2 [35]. Anti-

inflammatory properties of curcumin protected the liver of rats against injury caused by carbon tetrachloride (CCL_4) by dramatically suppressing serum levels of TNF-α, IL-6 and IFN-□. The doses of daily doses of curcumin used by these authors were higher than the doses used in our study, but the authors did not observe a dose-dependent effect. This may be related to the different experimental models or the maximum effective dose may have been reached.

Curcumin has also been shown to possess beneficial effects in alleviating arthritic symptoms in both animal and human models [38]. Indeed, there are clinical trials for the treatment of patients with rheumatoid arthritis and osteoarthritis with high doses of curcumin [39]. Due to their nontoxic and antimutagenic [40] nature, curcumin and other spice-derived principles hold promise in the treatment of arthritis. In animals models, carrageenan-induced paw inflammation is reduced in rats fed with curcumin at 30 mg/kg body weight [9]. Histological changes including infiltration of immune cells, synovial hyperplasia, cartilage destruction, and bone erosion in the hind paw sections were extensively suppressed by oral administration of curcumin (4 –100 mg/kg) in a dose-dependent manner [13]. MMP-1 and MMP-3, critical factors in the degradation of joint cartilages, whose levels were prominently increased in the vehicle-treatment group, were also reduced by curcumin. In regard to the modulation of intracellular signaling pathways, activation of JNK, but not ERK and p38 MAPK, was inhibited in a dose-dependent manner [13].

Although many studies [41-45] have investigated the effect of curcumin on modulation of different signaling pathways and transcription factors such as AP-1 and MAPKinases, the best-characterized and studied molecular target of curcumin is NF-κB. Inhibition of NF-κB may be one of main mechanisms for the anti-inflammatory effects of curcumin. In the present study, we demonstrated that curcumin inhibited the activation of NF-κB in ligature- induced periodontal disease in rats. Nevertheless, it is possible that the inhibition of pro-inflammatory gene expression by curcumin is partially dependent on the modulation of signaling pathways other than NF-κB. Even though we have not observed modulation of p38 MAPK, another signaling pathway involved in the expression of pro-inflammatory cytokines and bone resorption [46-52], it is important to bear in mind that our data is derived from protein from the multiple cell types present in the periodontal microenvironment. Curcumin may have differential effects on signaling pathways according to the cell type, e.g., macrophages, lymphocytes, fibroblasts and osteoblasts.

Besides its anti-inflammatory properties, curcumin is also investigated for its therapeutic potential in cancer, as an angiogenesis inhibitor in the treatment of corneal diseases [53] and in wound healing. Recent studies show that topical application of curcumin, incorporated in collagen vehicle accelerated healing in experimental surgical wounds of 2 cm^2, as indicated by enhanced cell proliferation and improved free radical scavenging in comparison with the wounds of negative control and collagen vehicle-treated rats [54]. In agreement with these reports, we observed a more organized and higher collagen content in periodontally diseased tissues of animals treated with both doses of curcumin in comparison to the tissues of the vehicle control group.

Curcumin has a long history as a traditional herbal medicine and has established anti-inflammatory properties in animals following systemic administration [37, 55, 56]. This compound is particularly interesting for therapeutic applications because its anti-inflammatory and anti-proliferative effects are potent, occurring at micromolar concentrations. Moreover, clinical trials have shown no significant toxicity even when administered at 8 g per day [39]. Although there are no reports of curcumin-induced toxicity associated with systemic administration, topical application may not only increase the potency of its effects but also prevent possible unwanted secondary effects in conditions associated with local chronic inflammation, such as periodontal diseases and rheumatoid arthritis. In summary, our results confirm and extend our prior in vitro observations (not shown) and suggest that curcumin might be a therapeutic anti-inflammatory candidate for the treatment of periodontal disease.

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Figure Legends

Figure 1 - Curcumin inhibits cytokine gene expression in experimental periodontitis. In the absence of periodontal disease, only PGE_2-synthase mRNA was significantly induced by the higher dose of curcumin in the absence of ligature-induced periodontal disease. The animals were treated with 30 or 100 mg/Kg of curcumin by oral gavage daily for 15 days. Control animals received the same volume of the vehicle by oral gavage. Cotton ligatures were placed around the first molars of rats bilaterally to induce periodontal disease. After 15 days, the animals were sacrificed and total RNA was isolated from gingival biopsies with ('Ligatures') and without ('No ligatures') periodontal disease and used for RT-qPCR performed with pre-designed primers and probes for the indicated target genes and to the housekeeping GAPDH using TaqMan reagents. The results were analyzed by the delta-Ct method and expression of target genes was normalized to GAPDH expression. (+) indicates significant reduction ($p< 0.05$) in comparison to vehicle control with ligatures. Bars indicate means and vertical lines standard error of mean of at least three animals in each experimental group.

Figure 2 – Effective inhibition of both IL-6 and TNF-α production in periodontally diseased tissues by curcumin. In the absence of ligatures, the higher dose of curcumin was associated to increased production of IL-6, whereas TNF-α production was not affected. Gingival tissues around both lower first molars with ('Ligature') and without ('No ligature') experimentally induced periodontal disease were collected after 15 days of administration of curcumin (30 or 100 mg/Kg) by oral gavage. Control animals received the same volume of vehicle. Total protein was extracted and used in ELISA tests to quantify the expression of the target cytokines. These tests were performed according to the manufacturer's instructions and the results for each sample were normalized to the concentration of total proteins determined by a Lowry-based microassay (DC assay, Bio-Rad). (+) indicates significant difference ($p< 0.05$) in

comparison to vehicle control in animals with ligature-induced periodontitis. Bars indicate means and vertical lines standard error of mean of at least three animals in each experimental group.

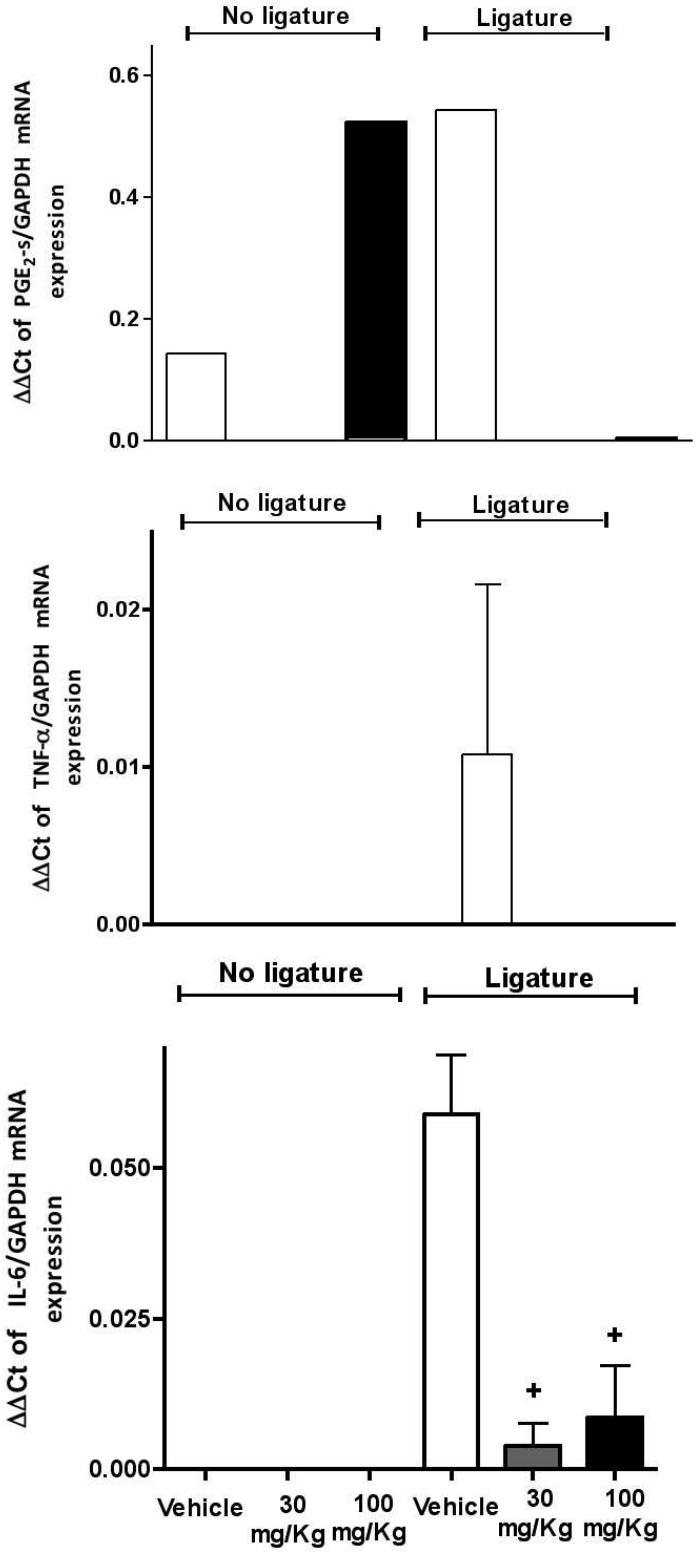
Figure 3 – Modulation of NF-kB and p38 MAPK activation in gingival tissues by systemic administration of curcumin. The animals were sacrificed after 15 days of curcumin (30 and 100 mg/Kg) or vehicle administration and the tissues surrounding the lower first molars were harvested. Total protein was isolated from gingival biopsies with a detergent-containing buffer (T-Per, Pierce) supplemented with protease and phosphatase inhibitor cocktails (Complete and Phos-Stop, Roche). Protein concentration was determined with a Lowry-based microassay (DC assay, Bio-Rad) and 60 μg of each sample were diluted in LDS-containing running buffer supplemented with DTT, heat-denatured, electrophoresed in 10% Tris-Acrilamide gels and transferred to 0.2 μM nitrocellulose membranes in a semi-dry transfer apparatus. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65 (NF-kB) and p38 MAPKinase. Expression levels of constitutive housekeeping GAPDH are shown to confirm equal protein loading. The images are representative of samples from three different animals in each experimental group.

Figure 4 – Stereometry analysis of the histological characteristics of gingival tissues subjected to ligature-induced periodontal disease according to the dose of curcumin (30 or 100 mg/Kg) administered for 15 days by oral gavage. Control animals were administered vehicle and negative control animals also were given vehicle but no ligature was placed around the first molars. A 50 x 50 μM grid was overlayed the histological images captured from H/E stained slides at 200 X magnification, allowing the analysis of an 'area of interest' of 2,500 μM^2. Two grids were used in each histological image: one was positioned 25 μM below the top of the alveolar bone crest,

and another at the base of the junctional epithelium, representing the ‘bone crest’ and ‘submarginal’ areas, respectively. The proportion of collagen, fibroblastic cells and inflammatory cells (distinguished by the morphological characteristics) in the area of interest was determined. A total of three images obtained from equally spaced slides (spanning 900 uM of the buccal-lingual aspect of the molars) were evaluated from each animal. Slides from at least three different animals in each experimental group were used. Both doses of curcumin markedly reduced the inflammatory infiltrate in periodontally-diseased tissues. The lower dose of curcumin (30 mg/Kg) induced a greater increase in the collagen content, whereas the higher dose (100 mg/Kg) was associated with increased proliferation of fibroblastic cells.

Figure 5 – Histological aspect of the gingival tissues according to the experimental group (control X ligature) and administration of curcumin (vehicle, 30 mg/Kg and 100 mg/Kg). Tissues were harvested 15 days after ligatures were placed and administration of curcumin was started. Semi-serial sections with 5 µM thickness were routinely processed and stained with hematoxylin and eosin. A total of three sections spaced 100 µM were evaluated for each first molar in a minimum of four animals in each experimental group. An epithelial layer of regular thickness, dense connective tissue with reduced number of cellular infiltrate and a smooth bone crest surface characterize the gingival tissues of control (non-ligated) animals (A). Placement of ligatures (B) produced an increase of the thickness of the epithelium layer, an intense cellular infiltrate and irregular bone crest surface with the presence of multinucleated osteoclast-like cells; whereas in animals treated with 30 mg/Kg (C) and 100 mg/Kg (D) of curcumin, the epithelial layer presents normal thickness and reduced cellular infiltrate. All images were obtained at 100 X magnification.

Figure 1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0.02
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30 mg/Kg
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Vehicle
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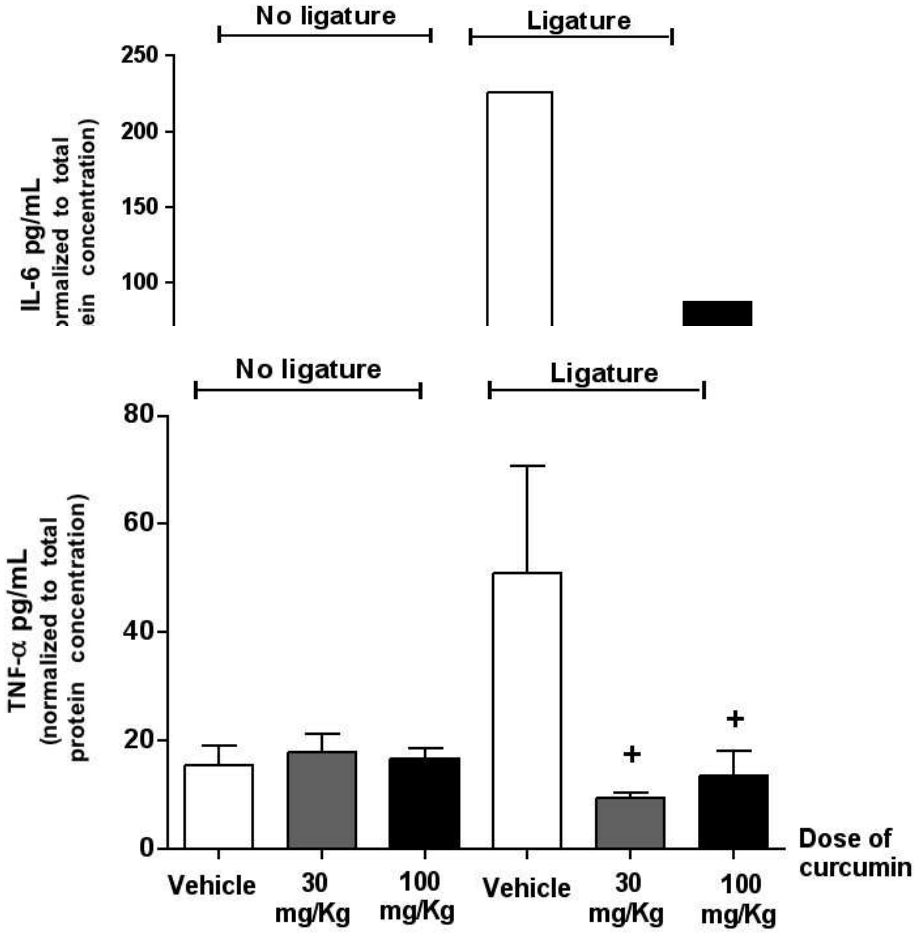
Figure 2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IL-6 pg/mL
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ein concentration)
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(normalized to total
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100 mg/Kg
Dose of curcumin

Figure 3

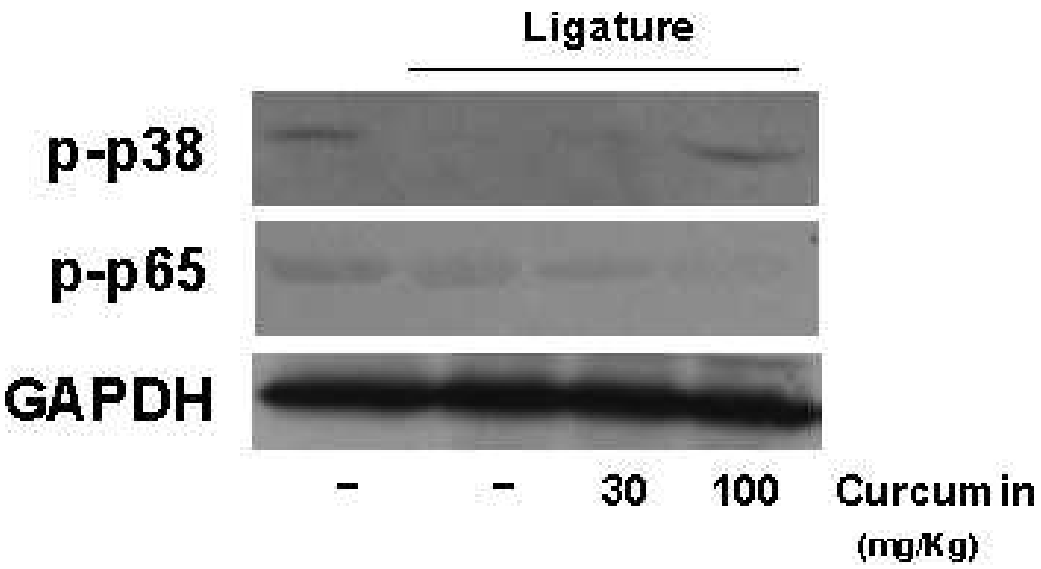
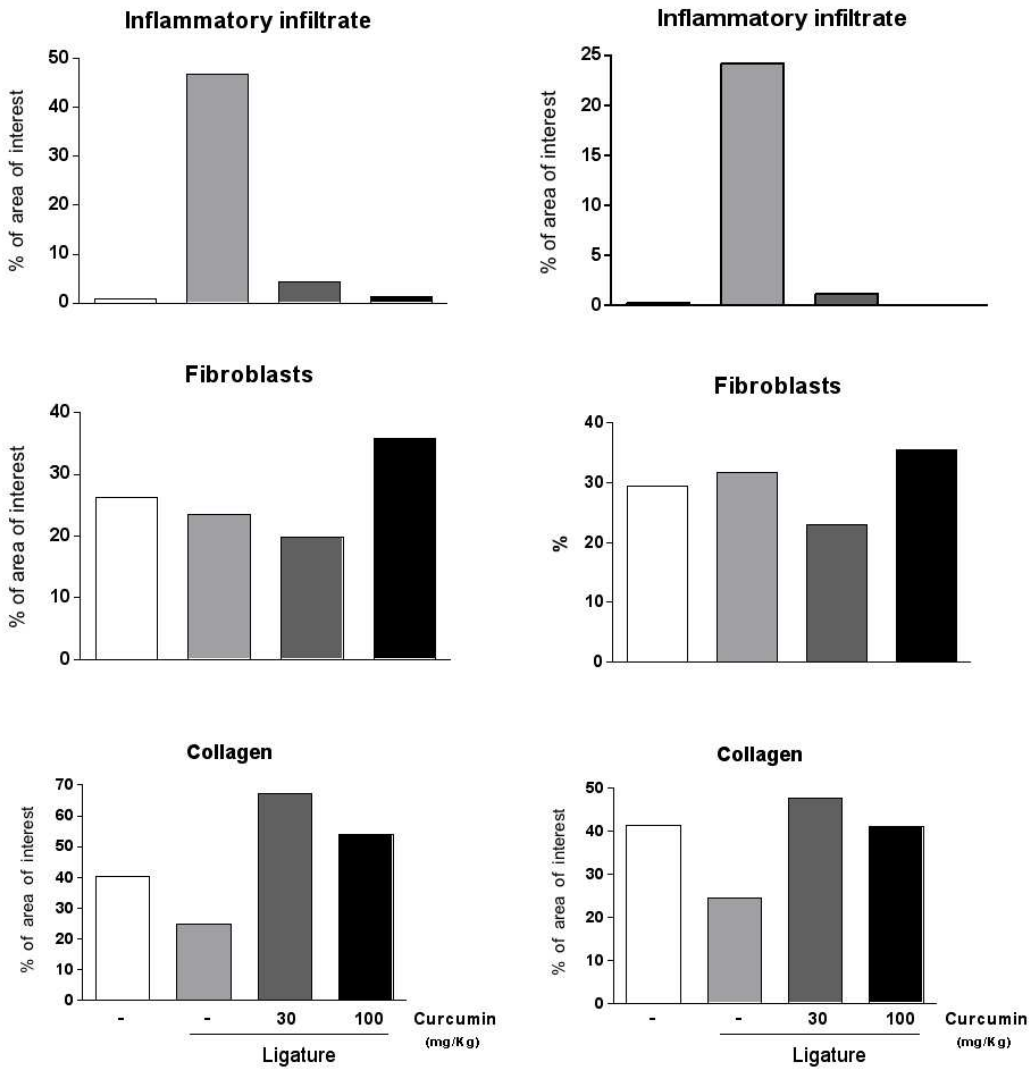


Figure 4


Inflammatory infiltrate
% of area of interest
50
40
30
20
10
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Inflammatory infiltrate
% of area of interest
25
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Fibroblasts
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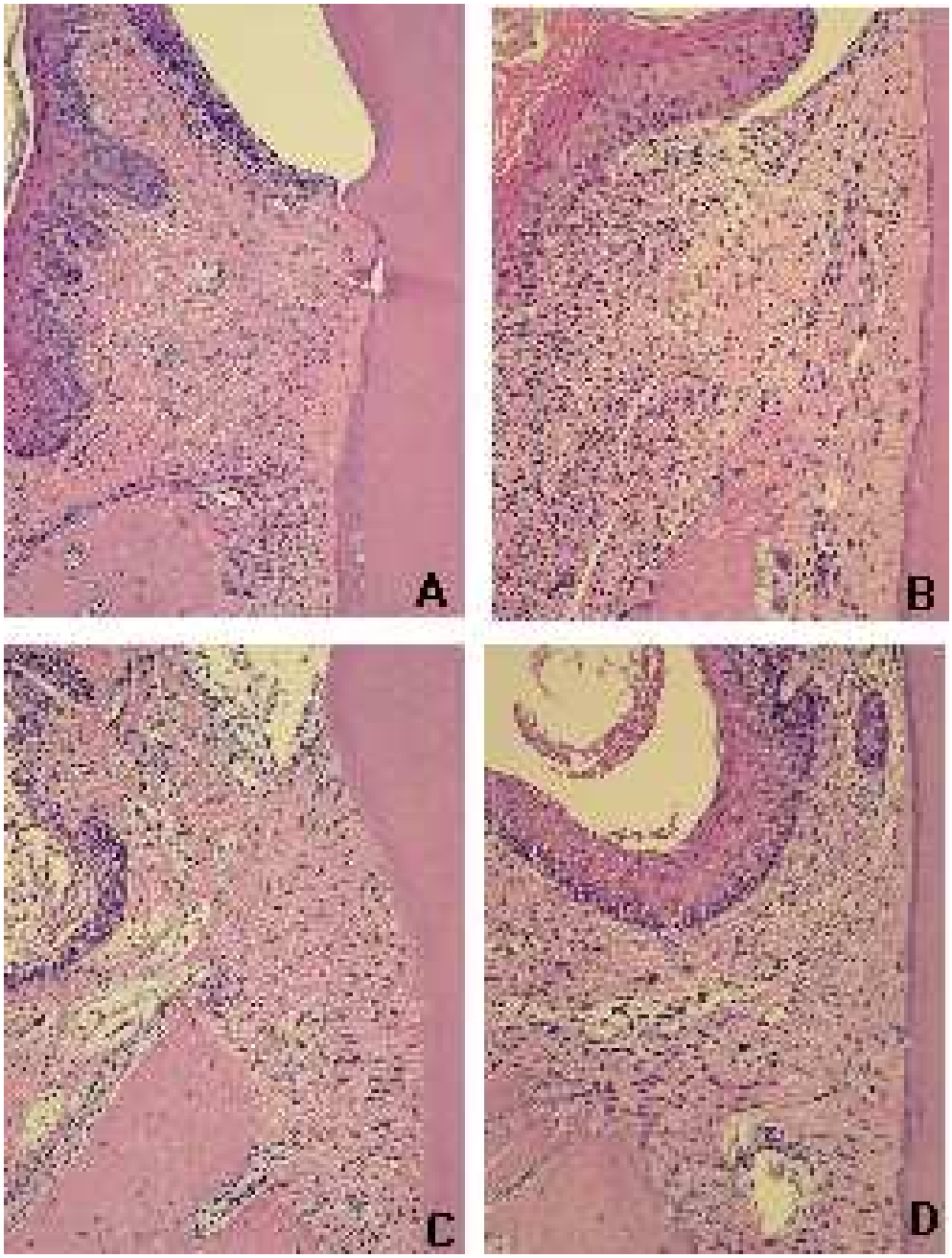
(mg/Kg)
Ligature

Figure 5



CAPÍTULO 3

Curcumin modulates the immune response associated with LPS-induced periodontal disease in rats

Running title: Curcumin inhibits LPS-induced periodontal disease

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Abstract
Curcumin is a plant-derived dietary spice with various biological activities. Its therapeutic applications have been studied in a variety of conditions, but not on periodontal disease. Periodontal disease is a chronic inflammatory condition initiated by an immune response to microorganisms of the dental biofilm. Experimental periodontal disease was induced in rats by injecting LPS in the gingival tissues on the palatal aspect of upper first molars (30 ug LPS, 3 times/week for 2 weeks). Curcumin was given to the rats by daily oral gavage in two doses (30 and 100 mg/Kg). RT-qPCR and ELISA were used to determine the expression of IL-6, TNF-alpha and PGE-2 synthase on the gingival tissues. The inflammatory status was evaluated by stereometric analysis, whereas modulation of p38 MAPk and NK-kB signaling was assessed by western blot. Curcumin effectively inhibited cytokine gene expression at mRNA and protein levels, but NF-kB was inhibited only with the lower dose of curcumin, whereas p38 MAPK activation was not affected. Curcumin produced a marked reduction on the inflammatory infiltrate and increased collagen content and fibroblastic cell numbers. Curcumin potently inhibits innate immune responses associated with periodontal disease, suggesting a therapeutic potential in this chronic inflammatoty condition.

Key-words: Curcumin, inflammation, innate immunity, periodontal disease

Introduction

The innate immune system in the periodontal tissues is successful in its protective role, given the limited number of bacteria actually invading the periodontal tissues relatively to the quantity of bacteria in the oral biofilm and the very rare occurrence of sepsis in spite of the bacterial load in the dental biofilm associated with periodontal disease. However, even though most of the microorganisms are located outside the host's periodontal tissues, their pathogen-associated molecular patterns (PAMPs) trigger an innate immune response by activating Toll-like receptor (TLR) signaling, which, in turn, may initiate and modulate adaptive immune responses (1, 2). These receptors are expressed by immune cells such as macrophages, neutrophils and dendritic cells as well as by non-immune resident cells, such as periodontal fibroblasts (3) and gingival epithelial cells (4). Within the periodontal tissues, expression of TLR2 and TLR4 is increased in severe disease states, suggesting that these receptors have an increased capacity to signal and influence downstream cytokine expression (5, 6).

Classically, TLR signaling is associated with the activation of innate immunity which initiates a series of cellular events, including production of cytokines and other biological mediators and phagocytosis that aim to eradicate the microbial aggression and restore homeostasis. Recently, the role of TLRs was expanded by the demonstration of host-derived ligands that indicate tissue destruction or cellular stress ('danger' signals) (7). The arbitrary/didactic boundaries between 'innate' and 'adaptive' arms of the immune response are also exposed by the fact that adaptive immune cells, such as T lymphocytes, also present functional TLRs (8, 9). Nevertheless, activation of innate immunity provides an immediate response to microbial stimuli and has the important role of eliciting and amplifying the more specific adaptive immune response.

The role of bacteria in the initiation and progress of periodontal disease is undisputed, and lipopolysaccharide (LPS) is one of the main pathogen-associated molecular patterns (PAMPs) that can stimulate the expression and production of proinflammatory cytokines through activation of TLRs. These cytokines are considered the main culprits in the degradation of soft and hard tissues that are the hallmarks of destructive periodontal disease. This critical role of LPS for periodontal diseases is demonstrated in the LPS-model of experimentally induced periodontal disease, in which direct injection of LPS into the gingival tissues initiates a local host response that involves recruitment of inflammatory cells, generation of prostanoids and cytokines, elaboration of lytic enzymes and activation of osteoclasts, culminating in the degradation of both soft and mineralized tissues of the periodontium (10, 11).

Curcumin, an extended pseudosymetric polyphenol extracted from plants and used as a dietary spice, has a variety of biological activities including anti-inflammatory properties, which have been partially attributed to direct interaction with protein targets (12). It has been found to modulate the growth and cellular response of various cell types involved in the immune response including T and B lymphocytes, macrophages, neutrophils, NK cells and dendritic cells (13).

Modulation of innate immunity is supported by the report that curcumin efficiently blocked LPS-induced expression of IL-12, IL-1β, IL-6 and TNF-α in dendritic cells. The same study showed that treatment of dendritic cells with curcumin before LPS stimulation completely suppressed the MAPK and nuclear translocation of NF-κB (14).

Curcumin inhibited the proliferation induced by concavalin A (Con A), phytohemagglutinin (PHA), and phorbol-12-myristate-13-acetate (PMA) of lymphocytes derived from fresh human spleen (15). During these studies, curcumin also

suppressed IL-12 synthesis; and IL-12 induced proliferation of lymphocytes. This correlated with suppression of NF-κB activation. Curcumin also has been reported to block Epstein-bar virus (EBV) induced immortalization of human B cells. This effect of curcumin appears to be mediated through downregulation of oxidative stress induced by cyclosporine and hydrogen peroxide (16). Apart from affecting normal B-cells, curcumin has been found to differentially reduce the proliferation of immature B-cell lymphoma (BKS-2) cells, but not of normal cells, by inducing apoptosis and this is associated with downregulation of Bcl-XL and the tumor suppressor gene p53 and almost complete inhibition of NF-κB activity (17). Curcumin could also increase cell death of refractory natural killer/T-cell lymphoma (NKTL) cell lines which are resistant to other therapies. This is direct linked to the suppression of the NF-κB and also blockage of Bcl-XL and activation of caspase-8 (18).

The effects of curcumin on these cell types suggest that it can regulate the immune response in vivo. The daily administration of curcumin (30mg/kg body weight/day) for 2 weeks in rats attenuated the ability of macrophages, stimulated with zymosan A for 18- 72h, to generate ROS (19) and secrete lysosomal enzymes collagenase, elastase, and hyaluronodase. (20). In other ex-vivo studies, curcumin was shown to downregulate Th1 cytokines and NO production by macrophages stimulated by AK-5 tumor cells, demonstrating the strong tumoricidal effect of curcumin and the important modulatory role of tumor-stroma interactions (21).

Some studies suggest that anti-inflammatory, chemo-preventive and other beneficial effects of certain dietary phytochemicals may be at least in part mediated through the modulation of TLR activation induced by endogenous molecules or chronic infection (22-24).

The immunomodulatory activity of curcumin can involve direct targeting of TLRs. For example, the cellular response to LPS is initiated by the interaction of LPS with the transmembrane complex including TLR4 and myeloid differentiation protein 2 (MD-2/TLR4). According to Gradisar *et al.* (22), curcumin binds with high affinity to MD-2 competing with LPS for the same binding site.

These immunomodulatory effects of curcumin can have important implications for the epidemiology, due to its widespread dietary use in some cultures, and treatment of infectious and non-infectious diseases associated with chronic inflammation (22). The therapeutic possibilities of this phytochemical are especially promising due to the lack of adverse effects associated with its consumption.

In this study we assessed the effect of curcumin administered by oral gavage on the expression of pro-inflammatory cytokines IL-6, TNF-α and PGE-2 and on the modulation of the signaling pathways NF-κB and p-38 in a model of LPS-induced periodontal disease in rats.

Methods

Experimental design

All the experimental protocols were approved by the Ethical Committee for Animal Experimentatuon (CEEA) of the School of Dentistry at Araraquara – UNESP and performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Forty male Holtzman rats (*Rattus norvegicus albinus*, Holtzman), weighing between 100 and 200 g, were randomly distributed into four experimental groups comprising 10 animals each. The rats were kept in a room with controlled temperature ($21 \pm 1^{o}C$) and humidity (65–70%) and a 12 h light–dark cycle.

General anesthesia was induced with intraperitoneal injections of ketamine and xylazine chlorydrate at 0.08 mL/100g body weight and 0.04 mL/100g body weight, respectively. 3 μl of a 10 mg/mL solution of *Eschericia coli* LPS (strain 055:B5 - Sigma Chem Co., St. Louis, MO, USA) diluted in PBS was injected into the palatal gingiva using a Hamilton micro-syringe (Agilent). The injections were done between the upper 1st and 2nd molars bilaterally three times a week for 15 days. Control animals received injections of the same volume of PBS vehicle. Administration of curcumin started the day before beginning of LPS injections for the induction of periodontal disease. Curcumin was administered daily by oral gavage in two different doses: one group received the lower dose of 30 mg/Kg/body weight, and the other group received 100 mg/Kg/body weight. Control animals were given the same volume of the corn oil vehicle.

After 15 days the animals were sacrificed and the maxillae were harvested and hemisected. Some of the block sections including 1^{st} and 2^{nd} molars with their surrounding tissues were submitted to routine histological processing to be used in the stereometry, whereas the soft tissues surrounding the 1^{st} molars were carefully dissected for extraction of RNA and protein. The remaining specimen was used for the assessment of bone loss. For the stereometric analysis, 3 specimens including the upper molars of the control group (treated with vehicle by oral gavage) were analyzed, whereas 4 specimens were analyzed for each of the experimental groups treated with curcumin. These tissue blocks were immersed directly in 10% buffered formalin fixative solution for 48 h and decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) during 2-3 months, under agitation at room temperature. Each specimen consisted of a section containing the 1st and 2nd molars and the surrounding alveolar process and soft tissues and was included in paraffin blocks. Serial sections of 4 μM

thickness were obtained in the bucco-lingual direction and stained with hematoxylin-eosin (H/E).

Stereometry

The analysis was conducted by a single examiner that was blind to the experimental groups using an optical microscope (Diastar Cambridge) set at 200 X magnification. Semi-serial sections of 4 μM were obtained from the tissue blocks on a buccal-lingual orientation. A total of 3 sections, spaced 100 μM from each other, were evaluated per tooth. A 50 x 50 μM grid was overlayed the histological images allowing the analysis of an 'area of interest' of 2,500 μM^2. Two grids composed of 5 x 5 10 μM squares were used in each histological image: one was positioned with its lower border 25 μM below the top of the alveolar bone crest and perpendicularly to the root surface, and the other was positioned with its upper border at the base of the junctional epithelium, representing the 'bone crest' and 'submarginal' areas, respectively. In both cases, the lateral border of the grids was always positioned over the most prominent part of the root surface in the area. Each one of the 25 points of the grid projected on each section was counted and the proportion of collagen, fibroblastic cells and inflammatory cells (distinguished by the morphological characteristics) in the area of interest was determined as percentage of the total points counted. A total of three images obtained from equally spaced slides (spanning 900 uM of the buccal-lingual aspect of the molars) were evaluated from each animal. Slides from at least three different animals in each experimental group were used.

Evaluation of cytokine gene expression at the mRNA level (RT-qPCR)

Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen Corp) according to the manufacturer's instructions. Concentration of RNA was determined by spectrophotometry and 700 ng of total RNA were reverse-transcribed into cDNA using

random hexamers as primers (High Capacity cDNA synthesis kit, Applied Biosystems). The relative abundance of the transcripts of the candidate inflammatory genes were measured by real-time reverse transcription-PCR (RT-qPCR) using Taqman chemistry and pre-designed sets of primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) on a StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate on a final reaction volume of 30 µL that included Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) for each target gene: TNF-α (tumor necrosis factor alpha), NM_013693; IL-6 (interleukin 6), NM031168; Ptgs-2 (prostaglandin-endoperoxide synthase-2), NM_011198; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), NM_008084; and cDNA template (corresponding to 30 ng of cDNA). Optimized thermal cycling conditions were: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 s and 60^{o}C for 1 min. For each sample, analyses of gene expression were performed in duplicate. The experiments were with samples isolated from at least three different animals in each experimental group. To normalize the amount of mRNA present in each reaction, the expression of GAPDH, which was not altered by the experimental conditions, was used as a housekeeping gene. To compare the expression levels among different samples, the relative expression level of the genes was calculated using the comparative ΔCT method using the thermocycler's software.

Activation of signaling pathways (Western-blot) and determination of cytokine gene expression at the protein level (ELISA)

Total proteins were extracted from gingival tissue samples using a detergent-based extraction buffer (T-PER, Tissue Protein Extraction Reagent – Pierce Biotechnology) containing a protease inhibitor cocktail (Protein Stabilizing Cocktail – Santa Cruz Biotechnology) according to manufacturer's instructions (Pierce Biotechnology). The

tissue samples were macerated in the buffer (50 μL / mg of tissue) and centrifuged for 5 min at 13,000 RPM at 4°C. The proteins were quantified using Lowry method (DC assay, Bio-Rad Laboratories) and 40 μg of total protein were added to a SDS sample buffer containing 2% SDS, DTT as a reducing agent, glycerol and bromophenol blue dye (Cell Signaling), heated-denaturated at 97° C for 5 min and chilled on ice of 5 min before loading on 10% SDS-polyacrylamide gels.

Electrophoresis on discontinuous acrylamide gels were carried out at 100 V for 90 min and subsequently electro-transferred to 0.2 μM nitrocellulose membranes using 300 mA constant current for 1 hour. The membranes were blocked for 1h in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 and subsequently washed for 5 min (3 times) with TBS-0.1% Tween at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C (1:100 dilution in PBS – phospho-p65 and phospho-p38 – Cell signaling). Membranes were washed in TBS-T buffer for 5 minutes (3 times) and incubated with secondary antibodies conjugated to horseradish peroxidase (1:1000 dilution in the blocking buffer) for 1 hour at room temperature, washed again with TBS-T buffer (5 min, 3 times). Detection of bands was carried out on radiographic film by using a chemiluminescence system (Lumi-Glo, Cell Signaling).

Proteins from these same samples were also used to in the ELISA assays to determine the concentration of PGE_2, IL-6 and TNF-α. These assays were performed according to the manufacturer's instructions (R&D Systems) and the results were normalized to the total concentration of protein in the samples. Samples from at least three different animals in each experimental group were used and assayed in duplicate.

Data analysis

The purpose of data analysis was to compare the results from curcumin-treated animals with those of vehicle treated control animals. Considering the two experimental groups treated with different doses of curcumin (30 and 100 mg/kg body weight) as independent variables, we used non-paired t-tests for the comparisons between all the groups (control x 30 mg/Kg of curcumin, control x 100 mg/Kg of curcumin and 30 x 100 mg/Kg of curcumin) with a significance level of 5%.

Results

Administration of curcumin did not change the weight or behavior of the experimental animals when compared to the animals treated with vehicle, suggesting the lack of serious adverse effects.

Curcumin effectively inhibits PGE2-synthase gene expression in LPS-induced periodontal disease

To evaluate the effect of curcumin on LPS-induced inflammatory gene expression the total RNA was extracted from periodontal tissues surrounding the upper first molars of rats treated with curcumin or vehicle both with and without LPS injections. PGE2-synthase (PGE2-s) is the murine homolog of human COX-2 and its expression was determined by RT-qPCR. Interestingly, the higher dose of curcumin (100 mg/Kg) induced PGE2-s mRNA expression in healthy gingival tissues. On the other hand, administration of curcumin completely abrogated LPS-induced PGE2-s mRNA expression in the gingival tissues. Expression of PGE2 at the protein level could not be detected in our samples (data not shown).

Curcumin abrogates LPS-induced IL-6 and TNF-alpha mRNA expression. Inhibition of IL-6 and TNF-alpha protein is associated with different doses of curcumin

Healthy and LPS-injected gingival tissues surrounding the upper first molars from animals treated with curcumin or vehicle were harvested and total RNA and protein were isolated and used for RT-qPCR and ELISA experiments, respectively. Similarly to the results for PGE2-s, administration of curcumin inhibited completely LPS-induced TNF-alpha and IL-6 mRNA expression in the gingival tissues. In fact, IL-6 expression was only detected in LPS-injected tissues, whereas TNF-alpha expression was not affected by curcumin in healthy gingival tissues. Inhibition of IL-6 protein was observed only in animals treated with the lower dose of curcumin (30 mg/Kg), whereas TNF-alpha was inhibited only in animals treated with the higher dose of curcumin (100 mg/Kg).

Only the lower dose of curcumin inhibited LPS-induced activation of NF-kB. Activation of p38 MAPK was not affected by curcumin.

Considering the prominent role of NF-kB and p38 MAPK on the expression of inflammatory genes downstream of TLR activation, we evaluated the effect of curcumin on the activation status of these two signaling pathways. LPS injections increased activation of NF-kB in the gingival tissues, which was markedly inhibited in the animals treated with the lower dose of curcumin (30 mg/Kg). The higher dose of curcumin (100 mg/Kg) did not change NF-kB activation status in the gingival tissues and neither the lower nor the higher dose had any effect on the activation of p38 MAPK.

Administration of curcumin reduced the number of inflammatory cells and increased the collagen content in the gingival tissues

Based on the evidence of anti-inflammatory properties of curcumin in other in vivo models and also in our own data demonstrating inhibition of PGE2-s, IL-6 and TNF-alpha, we then verified the changes on LPS-induced host response in the gingival

tissues associated with curcumin administration. In agreement with the modulation of inflammatory gene expression, administration of curcumin makedly inhibited LPS-induced host response in the gingival tissues. Stereometric analysis demonstrates that both doses of curcumin produced a significant reduction on the inflammatory infiltrate and also inhibited tissue destruction, as indicated by the increased collagen content in the gingival tissues of curcumin-treated animals.

Discussion

Curcumin has been shown to have a protective role in animal models of inflammatory diseases such as IBD, rheumatoid arthritis and colitis (25-32). This evidence, coupled with its low toxicity make curcumin a potentially viable supportive treatment option for chronic inflammatory conditions. Based on the evidence indicating that curcumin can markedly suppress the production of biological mediators that are hallmarks of inflammation and consequently various pathogenic processes, we investigated the effect of curcumin on the expression of proinflammatory mediators and host response in an LPS model of periodontal disease. We also assessed the effects of systemically administered curcumin on the activation status of NF-kB and p38 MAPK in the gingival tissues to gain further insight into the biological mechanism by which curcumin modulates the immune host response.

We found that administration of curcumin by oral gavage completely blocked PGE2 expression and produced a dose-dependent inhibition of IL-6 and TNF-alpha levels in the gingival tissues. The suppression of proinflammatory cytokines by curcumin was accompanied by a marked reduction of the inflammatory process verified by the stereometric analysis. These changes may be, at least partially, due to the dose-dependent inhibition of NF-kB activation in the gingival tissues of curcumin-treated

animals. Interestingly, administration of curcumin had no effect on LPS-induced p38 MAPK activation, which agrees with our observations in vitro using LPS-stimulated murine macrophages (data not shown - Guimaraes et al., submitted).

IL-6 and TNF-alpha play a relevant role in various conditions associated with chronic inflammation and appear to be important targets of curcumin. Intragastric administration of curcumin (100 mg/Kg) significantly reduced IL-6 and TNF-alpha serum levels in a sodium taurocholate infusion model of acute pancreatitis. However, this decrease of proinflammatory cytokine production in curcumin-treated animals was not accompanied by any changes on the histopathological scores of the pancreas, mesenteric lymph nodes, liver, lungs, spleen and kidneys (33). In contrast to these findings, and similarly to our observations in the LPS-injected gingival tissues, treatment with curcumin improved pancreatic histology in two models of experimental pancreatitis in rats (34). In this study, IL-6, TNF-alpha and iNOS expression were all reduced in curcumin-treated animals. The authors suggest that these effects of curcumin may be, at least in part, mediated by inhibition of NF-κB and AP-1, transcription factors with prominent roles in the expression of inflammatory mediators, which are also considered to have a critical role in the early events in experimental pancreatitis. According to Gulcubuk *et al*. (33) the lack of improvement on pancreatic injury in curcumin-treated animals in their study and the contrast with the study by Gukovsky *et al.* (34) may be associated with differences in the experimental models used, the observation period and the cytokine level, which did not drop to the basal level. Sodium taurocholate induces severe hemorrhagic necrotizing pancreatitis, while other inductive agents, such as cerulean, result in mild edematous pancreatitis. Other important factor to be considered is the time period between the induction of

pancreatitis and the killing. Gulcubuk's study the animals were killed on day 6, but Gukovsky *et al.* (34) sacrificed the animals at 6 h.

In another study, curcumin administered by daily oral gavage decreased TNF-alpha and IL-6 levels in the liver of rats in a carbon tetrachloride (CCL_4)-induced model of liver injury (35). The doses (200 and 400 mg/Kg) and duration of treatment (8 weeks) were both greater than those used in the present study (30 and 100 mg/Kg for 2 weeks), but the decrease on inflammatory gene expression were also associated with improved histopathological characteristics of the liver in curcumin-treated animals, again similarly to our observations in the LPS model of periodontitis. Numerous studies demonstrated that certain phytochemicals including polyphenols possessing anti-inflammatory effects inhibit NF-κB and MAPKinase signaling downstream of various receptors, including TNF-alpha receptor and TLR4 (36). Curcumin inhibited the induction of nitric oxide synthase induced by LPS/IFNγ in Raw 264.7 cells (37) and suppressed LPS-induced COX-2 gene expression by inhibiting NF-κB and AP-1 in microglial cells (38). In a rabbit model of sepsis, curcumin reduced LPS-induced fever by attenuating the expression of IL-1β, IL-6 and TNF-alpha in the serum due to suppression of NF-κB activation (39).

Activation of NF-kB is known to be pivotal for the expression of inflammatory cytokines involved in the pathogenesis of various inflammatory diseases (40, 41), which turns it into a major target for host modulation therapeutic strategies. Activation of NF-kB was also observed in oral epithelial cells exposed to the periodontopathogens *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (42), indicating the crucial role of NF-kB on innate immunity in the oral cavity. Indeed, induction of gene expression in human monocytic cell line by *Porphyromonas gingivalis* LPS was abolished by inactivation of IKK/NF-kB (43). In another study, inhibition of NF-kB

activation by endocannabinoids, an emerging class of lipid mediators with immunosuppressive and anti-inflammatory effects, derived from arachidonic acids and found in several tissues, reduced the production of proinflammatory mediators (IL-6, IL-8 and MCP-1) induced by *Porphyromonas gingivalis* LPS in human gingival fibroblasts (44), another resident cell type with an important role in innate immune response in periodontal diseases. Taken together, these studies indicate a role of NF-kB on the expression of fundamental mediators in periodontal disease (45). There is plenty of evidence indicating that NF-kB is one of the main targets of curcumin, and in this study we show that the potential effects of curcumin on periodontal disease may be, at least in part, due to the modulation of NF-kB activation downstream of TLR4 activation by LPS.

Inhibition of LPS signaling by curcumin may have important implications for treatment and epidemiology of chronic inflammatory diseases caused by bacterial infections (22). Moreover, it is now recognized that inflammation plays a fundamental role in many chronic diseases including cancer, atherosclerosis and rheumatoid arthritis. Some studies suggest that anti-inflammatory, chemopreventive and other beneficial effects of certain dietary phytochemicals may to be associated with the modulation of inflammation by interfering with TLR activation by PAMPs and endogenous molecules (22, 24).

In our study, LPS-induced NF-kB activation in the gingival tissues was inhibited only with the lower dose of curcumin. However, both low and high doses of curcumin produced a marked decrease on inflammation and a significant decrease of the expression of the proinflammatory mediators PGE-2, TNF-alpha and IL-6. This suggests that curcumin may modulate other signaling pathways downstream of TLR activation that are also relevant for the expression of these cytokines. One signaling

pathway that is relevant for the expression of inflammatory mediators and could be a candidate for a 'NF-kB alternative' is p38 MAPK; however, in our experimental model, p38 activation was not modulated by curcumin.

In summary, our results demonstrate a potent anti-inflammatory activity of orally administered curcumin in a model of LPS-induced periodontal disease and suggest a potential therapeutic role for curcumin in the treatment of this condition. Future studies will aim to characterize the spectrum of the biological effects of curcumin in this in vivo model and also in vitro, in relevant cells of the immune response associated with periodontal disease by using a focused array approach.

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Figure legends

Figure 1 - Curcumin inhibits cytokine gene expression induced by LPS in gingival tissues. Animals were treated with 30 or 100 mg/Kg of curcumin by oral gavage daily for 15 days. Control animals received the same volume of the vehicle by oral gavage. 30 μg of LPS or an equivalent volume of PBS vehicle were injected in palatal aspect of the gingival tissues around the upper first molars three times/week for 2 weeks. Total RNA was isolated from gingival biopsies with ('LPS') and without ('PBS control') experimental periodontal disease and used for RT-qPCR performed with pre-designed primers and probes for the indicated target genes and to the housekeeping GAPDH using TaqMan reagents. In the tissues injected with PBS vehicle only PGE_2-synthase mRNA was detectable and, interestingly, the higher dose of curcumin increased its expression. TNF-alpha and IL-6 mRNAs were detected only in LPS-injected tissues of animals that received the vehicle by oral gavage, indicating that both doses of curcumin were able to suppress completely the expression of these cytokines in the gingival tissues. All samples were assayed in duplicate and the results were analyzed by the delta-Ct method and expression of target genes was normalized to GAPDH expression. (*****) indicates significant reduction ($p < 0.05$) in comparison to vehicle control in LPS-injected tissues. Bars indicate means and vertical lines standard error of mean of at least three animals in each experimental group, except for PGE2-s mRNA analysis in which the samples of three animals in each experimental group were pooled to enable detection and there are no error bars.

Figure 2 – Effect of curcumin administration on IL-6 and TNF-α protein production in gingival tissues with and without LPS injections. Total protein was extracted and used in ELISA tests to quantify the expression of the target cytokines. These tests were performed according to the manufacturer's instructions and the results for each sample

were normalized to the concentration of total proteins determined by a Lowry-based microassay (DC assay, Bio-Rad). In control, healthy gingival tissues injected with PBS vehicle, curcumin had no effect on TNF-alpha production, whereas IL-6 was undetectable. Both IL-6 and TNF-alpha production was induced in LPS-injected tissues and the lower dose (30 mg/Kg) was more effective in the inhibition of IL-6, whereas there was a trend for a dose-dependent inhibition of TNF-alpha by curcumin. (+) indicates significant difference ($p < 0.05$) in comparison to vehicle control in animals with ligature-induced periodontitis. For TNF-alpha production, bars indicate means and vertical lines standard error of mean of at least three animals in each experimental group. IL-6 concentration was determined in pooled samples from three animals in each experimental group due to the low level of expression, and the bar indicate the average of the triplicate measurement from these pooled samples.

Figure 3 – Curcumin inhibits activation of NF-kB, but not of p38 MAPK, by LPS injections in the gingival tissues. The animals were sacrificed after 15 days of curcumin (30 and 100 mg/Kg) or vehicle administration and the tissues surrounding the upper first molars were harvested. Total protein was isolated from gingival biopsies with a detergent-containing buffer (T-Per, Pierce) supplemented with protease and phosphatase inhibitor cocktails (Complete and Phos-Stop, Roche). Protein concentration was determined with a Lowry-based microassay (DC assay, Bio-Rad) and 60 μg of each sample were diluted in LDS-containing running buffer supplemented with DTT, heat-denatured, electrophoresed in 10% Tris-Acrilamide gels and transferred to 0.2 μM nitrocellulose membranes in a semi-dry transfer apparatus. Activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65 (NF-kB) and p38 MAPKinase. NF-kB was clearly inhibited in the tissues of animals treated with the lower dose (30 mg/Kg) of curcumin, whereas activation status of p38 MAPK was not

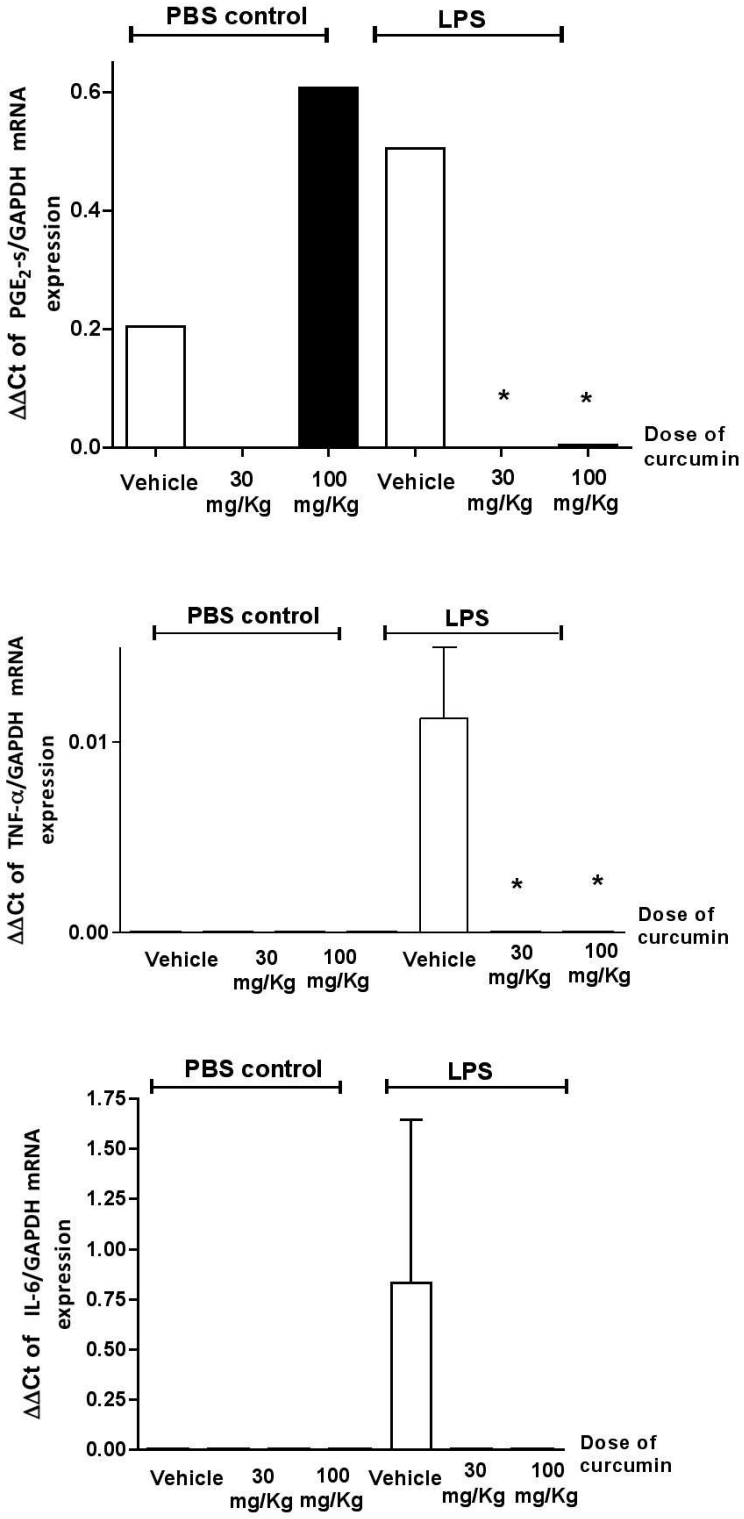
changed by either dose of curcumin. Expression levels of constitutive housekeeping GAPDH are shown to confirm equal protein loading. The images are representative of samples from three different animals in each experimental group.

Figure 4 – Stereometry analysis of gingival tissues injected with LPS, according to the experimental group: vehicle control, 30 or 100 mg/Kg of curcumin administered for 15 days by oral gavage. Negative control (PBS injections and vehicle administration by oral gavage) were analyzed for comparative purposes. A 50 x 50 μM grid composed of 5 x 5 10 μM squares was overlayed the histological images captured from H/E stained slides at 200 X magnification, allowing the analysis of an 'area of interest' of 2,500 μM^2. Two grids were used in each histological image: one was positioned 25 μM below the top of the alveolar bone crest, and another at the base of the junctional epithelium, representing the 'bone crest' and 'submarginal' areas, respectively. The proportion of collagen, fibroblastic cells and inflammatory cells (distinguished by the morphological characteristics) in the area of interest was determined. A total of three images obtained from equally spaced slides (spanning 900 uM of the buccal-lingual aspect of the molars) were evaluated from each animal. Slides from at least three different animals in each experimental group were used. Both doses of curcumin markedly reduced the inflammatory infiltrate in LPS-injected gingival tissues. Curcumin also increased the number of fibroblastic cells and the collagen content in the tissues.

Figure 5 – Histological aspect of the gingival tissues according to the experimental group (PBS X LPS injections) and administration of curcumin (vehicle, 30 mg/Kg and 100 mg/Kg). Tissues were harvested 15 days after LPS or PBS injections and administration of curcumin was started. Semi-serial sections with 5 μM thickness were routinely processed and stained with hematoxylin and eosin. A total of three sections spaced 100 μM were evaluated for each first molar in a minimum of four animals in

each experimental group. A thick keratinized epithelium layer corresponding to the palatal gingival with an underlying dense connective tissue with reduced number of cellular infiltrate and a smooth bone crest surface characterize the gingival tissues of control (PBS-injected) animals (A). LPS injections (B) produced a decrease on the thickness of the epithelium layer and an intense cellular infiltrate and irregular bone crest surface with the presence of multinucleated osteoclast-like cells; whereas in animals treated with 30 mg/Kg (C) and 100 mg/Kg (D) of curcumin, the cellular infiltrate was markedly reduced and the collagen content was increased in comparison with the LPS-injected sites (B). Images were obtained at 100 X magnification and the insert depicts the alveolar bone crest area at 200 X magnification. These images are representative of the histological aspect observed in at least four animals in each experimental group.

Figure 1


PBS control
LPS
ΔΔCt of PGE2-s/GAPDH mRNA expression
0.6
0.4
0.2
0.0
*
*
Dose of curcumin
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30 mg/Kg
100 mg/Kg
Vehicle
30 mg/Kg
100 mg/Kg
PBS control
LPS
ΔΔCt of TNF-α/GAPDH mRNA expression
0.01
0.00
*
*
Dose of curcumin
Vehicle
30 mg/Kg
100 mg/Kg
Vehicle
30 mg/Kg
100 mg/Kg
PBS control
LPS
ΔΔCt of IL-6/GAPDH mRNA expression
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Dose of curcumin
Vehicle
30 mg/Kg
100 mg/Kg
Vehicle
30 mg/Kg
100 mg/Kg

Figure 2

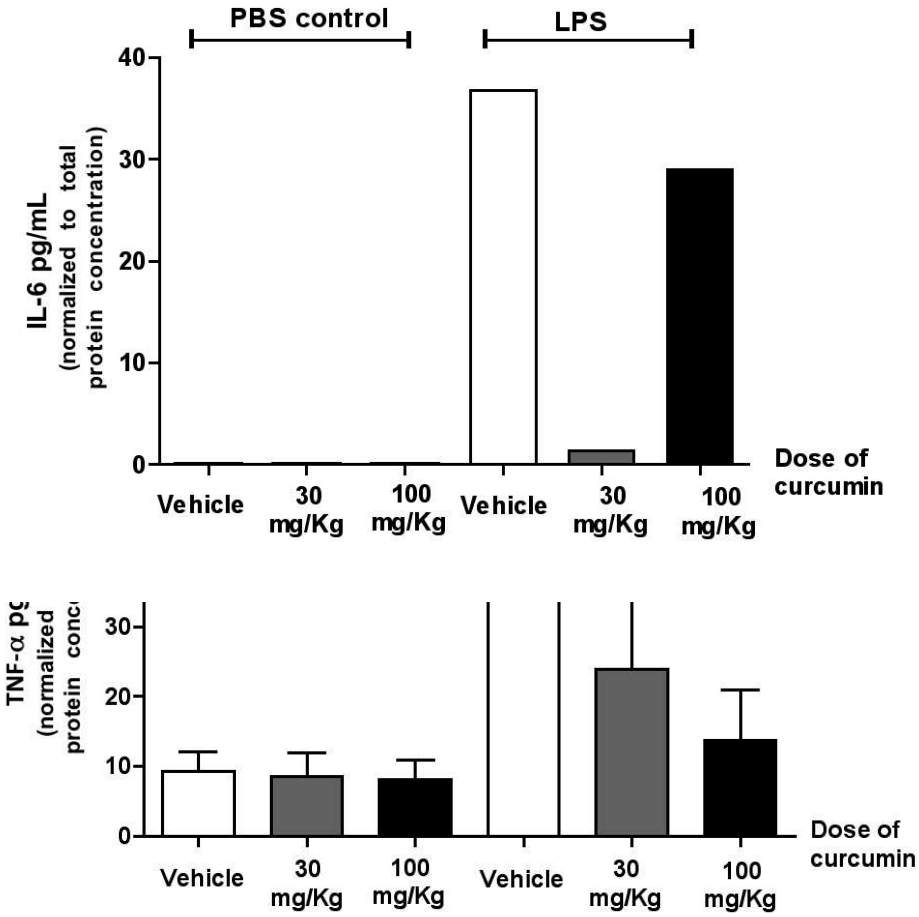

PBS control
LPS
IL-6 pg/mL
(normalized to total protein concentration)
40
30
20
10
0
Vehicle
30 mg/Kg
100 mg/Kg
Vehicle
30 mg/Kg
100 mg/Kg
Dose of curcumin
30
20
10
0
Vehicle
30 mg/Kg
100 mg/Kg
Vehicle
30 mg/Kg
100 mg/Kg
Dose of curcumin

Figure 3

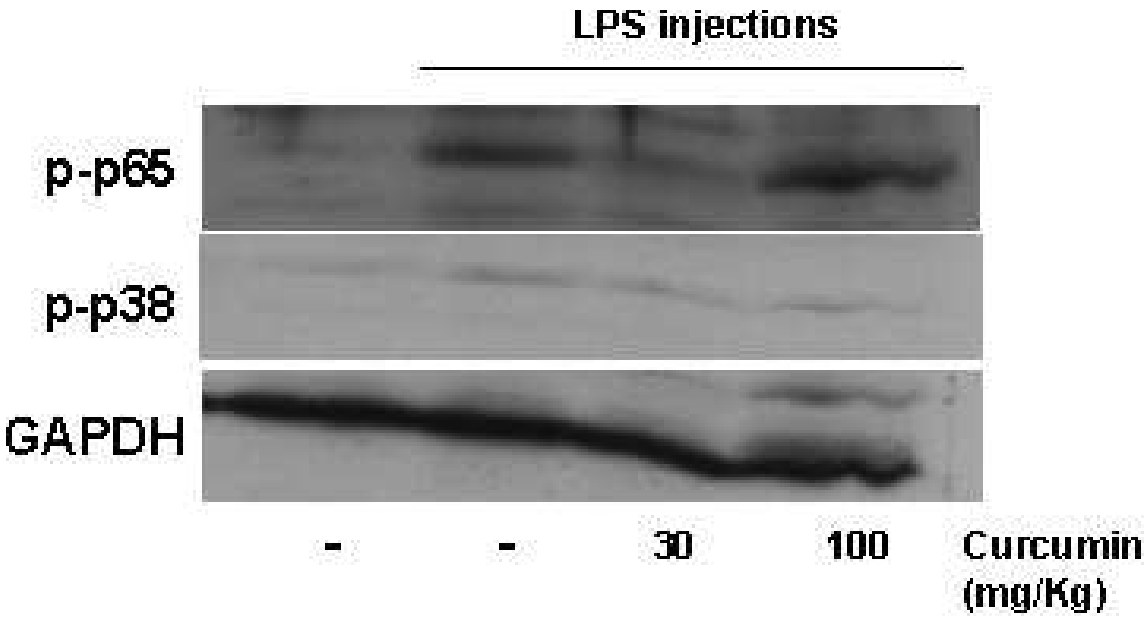


Figure 4

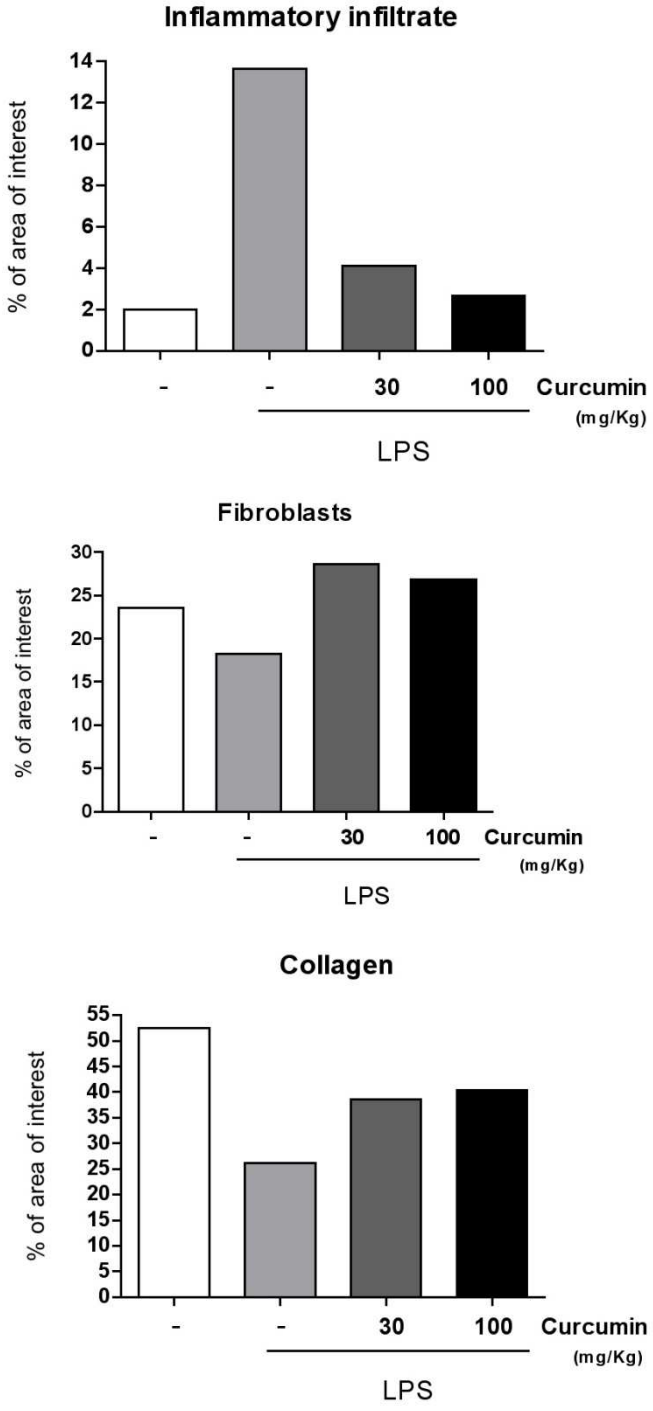
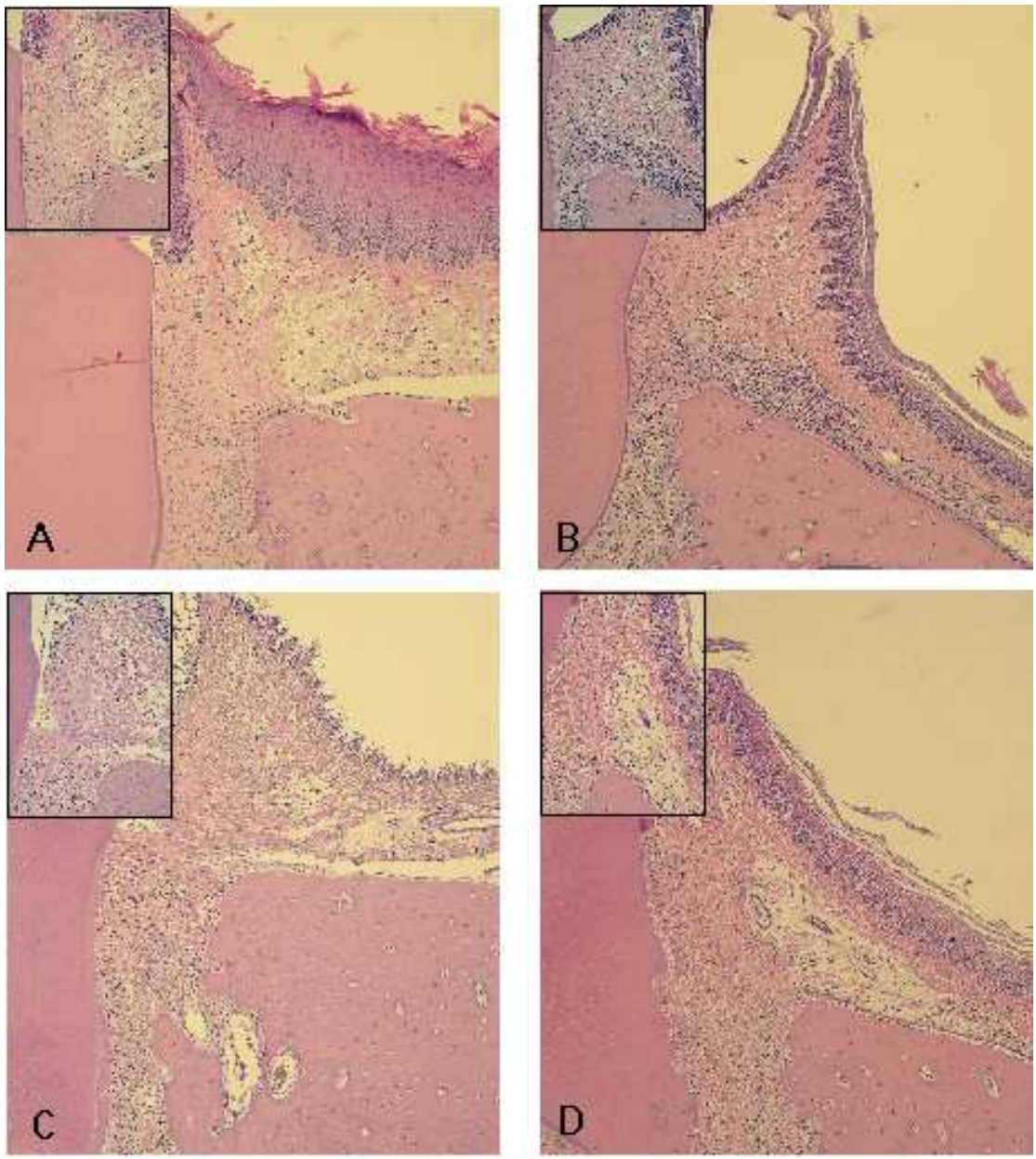

Inflammatory infiltrate
% of area of interest
14
12
10
8
6
4
2
0
-
-
30
100
Curcumin
(mg/Kg)
LPS
Fibroblasts
% of area of interest
30
25
20
15
10
5
0
-
-
30
100
Curcumin
(mg/Kg)
LPS
Collagen
% of area of interest
55
50
45
40
35
30
25
20
15
10
5
0
-
-
30
100
Curcumin
(mg/Kg)
LPS

Figure 5



MATERIAL E MÉTODO

Material e Método

Linhagem celular e materiais

Macrófagos de camundongo, Raw 264.7, foram cultivados em DMEM suplementado com 100 μg/mL de streptomicina, e 10% de soro fetal bovino inativado pelo calor e mantidos em atmosfera úmida a 37°C e 5% CO_2. Lipopolissacarídeo (LPS) de *Escherichia coli* (strain 055: B5 # 6529) e curcumin (# C1386) foram obtidos comercialmente da Sigma Chemical Co. Inibidores bioquímicos de p38 (SB203580, # 559389) e NF-kB (Bay 11-7082, # 196870) foram obtidos da Calbiochem. LPS de *E. coli* foi diluído em PBS a 1 mg/ml e usado em uma concentração final de 1 μg/ml; curcumin e os inibidores bioquímicos foram diluídos em DMSO. Anticorpos policlonais de coelho contra as formas fosforiladas de p38 MAP kinase (# 9211), NF-κB (p65) (# 3033), JNK (# 9251) e ERK1/2 (# 4376) foram provenientes da Cell Signaling, bem como os anticorpos secundários conjugados à HRP.

Viabilidade Celular

O efeito de curcumin e do inibidor de NF-kB sobre a viabilidade celular foi determinado usando um kit comercial (Cell Titer 96 Aqueous; Promega Corp.) de acordo com as instruções do fabricante. Este kit avalia a viabilidade celular através da redução do componente tetrazolium em formazan por enzimas desidrogenases presentes em células metabolicamente ativas. 1 x 10^5 células foram plaqueadas em cada poço de placas de cultura de 96 poços. Após um período de 18 horas, para permitir adesão celular à placa, as células foram lavadas uma vez com PBS e então desinduzidas com meio de cultura com concentração reduzida de FBS (0,3%). Curcumin foi adicionado ao meio de cultura em varias concentrações para um experimento dose-resposta e as células incubadas por 24 horas. Controles foram representados pelo mesmo volume do

veiculo DMSO e também por diferentes concentrações do inibidor de NF-kB (Bay 11-7082). 20 µl do reagente contendo o sal de tetrazolium (MTS) foi adicionado a cada poço, incubado por 1 e 2 horas e os resultados obtidos através da medição da absorbância a 490 nm em um leitor de microplacas (Spectramax L, Molecular Devices). O número de células viáveis nos poços tratados com curcumin ou Bay 11-7082 foi estimado em relação ao controle tratado com o veiculo.

Obtenção e manutenção dos animais

Foram utilizados 40 ratos Holtzman (Rattus norvegicus albinus Holtzman) machos e adultos, mantidos no biotério da Faculdade de Odontologia de Araraquara, de acordo com o Colégio Brasileiro de Experimentação Animal (COBEA) e o Comitê de Ética em experimentação Animal (CEEA) desta instituição. O protocolo experimental foi submetido e aprovado pelo Comitê de Ética em Experimentação Animal (CEEA) da Faculdade de Odontologia de Araraquara – UNESP (Anexo 1). Os animais foram acomodados em gaiolas de polipropileno e receberam alimentação granulada (Labina/Purina) e água *ad libitum*, sendo pesados semanalmente.

Os 40 animais foram distribuídos em 4 grupos experimentais: controle negativo (10 animais) que não receberam qualquer manipulação, incluindo indução da doença periodontal por ligadura ou LPS, tampouco administração de curcumin, apenas recebendo injeção do veiculo (PBS, pH 7.4) utilizado na diluição do LPS microbiano No grupo experimental (30 animais), estão os animais que foram submetidos à indução de doença periodontal com ligaduras nos molares inferiores e injeção de LPS na região palatina. Os 30 animais do grupo experimental foram subdivididos em 3 três grupos de 10 animais, sendo que dois deles receberam doses de 30 mg/kg/ peso corporal de curcumin, e o outro 100mg/kg/peso corporal via oral, durante todo o período experimental. O

grupo restante recebeu o mesmo volume do veículo utilizado na diluição do curcumin via oral (controle).

Administração de curcumin e indução da doença periodontal

Curcumin foi dissolvido em óleo de milho e administrado aos animais via oral, diariamente, nas dosagens de 30 mg/Kg ou 100 mg/Kg de peso corporal uma vez ao dia durante os 15 dias do experimento.

No dia seguinte ao inicio da administração do curcumin os animais foram submetidos à indução da doença periodontal. Neste estudo, o modelo de indução de doença periodontal por ligadura, assim como o de injeção de LPS na região palatina, foi utilizado.

Inicialmente os animais receberam anestesia geral (0,08 mL de Ketamina e 0,04 mL de cloridrato de xilazina por 100 g de peso corporal) e foram em seguida posicionados em mesa operatória. O modelo de ligadura foi obtido com a colocação de um fio de algodão número 24 ao redor dos primeiros molares inferiores bilateralmente. Na região palatina entre o 1º e 2º molar, realizou-se, com uma microseringa tipo Hamilton (Agilent), a injeção de um volume de 3 uL, correspondendo a um total de 30 μg, de LPS de *Eschericia coli*. As injeções de LPS foram repetidas 03 vezes por semana bilateralmente, durante as duas semanas de duração do experimento, sendo os animais sedados previamente à realização das injeções. O grupo controle recebeu injeções de PBS (veículo) na gengiva palatina dos primeiros molares superiores, enquanto ligaduras não foram colocadas nos molares inferiores.

Sacrifício dos animais, obtenção de amostras e delineamento experimental

Após 15 dias da colocação das ligaduras e inicio das injeções de LPS todos os animais foram sacrificados. O período de 15 dias foi selecionado por ser representativo

da doença instalada em nível moderado/avançado de severidade, como indicado por estudos anteriores de nosso grupo de pesquisa [33, 45]. A cronologia do experimento está representada na Tabela 2.

Tabela 2 – Cronologia do Experimento

	- 1	0	2	5	7	9	11	15
Início administração curcumin	+							
Colocação ligadura		+						
Injeção LPS		+	+	+	+	+	+	
Checagem ligaduras			+	+	+	+	+	+
Sacrifício								+

+ significa que o procedimento foi realizado no período indicado.

Imediatamente após o sacrifício, a mandíbula de cada animal foi removida e separada em duas hemimandíbulas, assim como os blocos envolvendo 1^{o} e 2^{o} molares superiores. Um número de 3 pecas superiores e 3 pecas inferiores dos animais sacrificados foi destinado a análise histológica e imunohistoquímica, 5 pecas inferiores foram destinadas a análise por microtomografia computadorizada e 5 peças superiores destinadas a verificação da perda óssea por meio da coloração com azul de metileno, e o restante das pecas destinado a coleta de tecidos moles para extração de RNA total e proteínas.

As peças para estereometria e imunohistoquímica foram fixadas em formol tamponado a 10% durante 48h e então desmineralizadas em solução de EDTA (0.5 M, pH 8.0) sob agitação à temperatura ambiente durante 2-3 meses (com troca da solução 2x/semana), com posterior inclusão em parafina. Cortes seriados de 4 µm de espessura foram obtidos na direção vestíbulo-lingual, montados em lâminas de vidro e aquelas

destinadas à analise estereométrica do processo inflamatório receberam a coloração (H/E). Os tecidos periodontais coletados para extração de RNA e proteína total foram armazenados em nitrogênio líquido logo após sua remoção e em seguida transferidos para o freezer -80°C até o momento do processamento para as análises.

Análises bioquímicas:

No momento do sacrifício, após anestesia dos animais, 1 mL de sangue de todos os animais dos grupos experimentais foi coletado por punção cardíaca. O sangue foi homogenizado e centrifugado a 1000 rpm durante 10 minutos para separação do soro. Posteriormente este soro foi aliquotado e encaminhado ao Departamento de Análises Clinicas da Faculdade de Ciências Farmacêuticas de Araraquara - UNESP, para realização dos testes bioquímicos.

Níveis séricos de cálcio e fósforo foram avaliados através da utilização dos kits (Ca Arzenato Liquiform 95 – Labtest Diagnóstica) e (Fosfóro UV Liquiform 12 - Labtest Diagnóstica) respectivamente, de acordo com as instruções do fabricante.

Estereometria

A análise estereométrica do processo inflamatório foi realizada com o auxílio de um microscópio de luz (Diastar-Cambridge Instruments) na magnificação de 200 x. Um total de 3 cortes por dente foi avaliado com um intervalo 100 μM entre esses cortes. A técnica estereológica de contagem de pontos foi empregada nos cortes corados com hematoxilina e eosina (H/E) para avaliar a proporção de componentes teciduais em um plano bidimensional por microscopia. Este procedimento permite a avaliação quantitativa do quadro inflamatório nas proximidades da agressão.

Com esta finalidade, foi determinada a densidade volumétrica relativa (ou proporção) dos seguintes componentes teciduais: fibras colágenas, fibroblastos, outros tipos de células (inflamatórias, osteoclastos, cementoblastos, etc.), vasos sanguíneos e outras estruturas (que correspondem ao tecido ósseo, epitelial, dente e nervos). Duas diferentes localizações anatômicas da porção vestibular e/ou palatina dos cortes foram quantificadas:

1- o tecido conjuntivo subjacente ao sulco gengival, junto à base do epitélio juncional (EJ) – O limite coronal foi representado pela borda apical do epitélio juncional e a estrutura dental correspondeu ao limite medial.

2- o tecido conjuntivo próximo ao topo da crista óssea, junto à superfície dental (CO) – O limite apical foi delimitado 25 μM abaixo da porção mais coronal da crista óssea e a estrutura dental correspondeu ao limite medial.

Nos cortes que receberam injeções de LPS, a análise considerou apenas a região palatina. Após a captura de imagens, a quantificação foi feita com o auxílio de grades confeccionadas com a dimensão de 50 μM x 50 μM ou 2500 μM^2 de área (5 x 5 quadrados de 10 μM de lado). Estas grades foram posicionadas (sobrepostas) nas imagens digitalizadas dos cortes histológicos preenchendo as 2 regiões de interesse da imagem (EJ e CO) e, para cada grade, foram contados 25 pontos coincidentes sobre as estruturas histológicas. Em seguida, foi feita uma análise percentual de cada componente tecidual em relação ao número total de pontos contados por período experimental baseado nos trabalhos de [80, 94] para a representação gráfica da estereometria.

Imunohistoquímica

Para imunolocalização e avaliação do estado de ativação de NF-kB nos tecidos gengivais, os cortes histológicos foram desparafinizados em xilol, rehidratados e

posteriormente incubados por 5 minutos com solução de peróxido de hidrogênio 3% para bloqueio da peroxidase endógena. Os cortes foram submetidos à etapa de recuperação antigênica com tripsina 0,5% a 37^{o}C durante 10 minutos e deixados sobre a bancada para resfriamento por mais 10 minutos. Em seguida os cortes foram incubados com anticorpo policlonal de coelho anti-NF-kB p50 (Santa Cruz Biotechnology, sc-114) diluído 1:250 em BSA 1%, e deixados por 18 h (‘*overnight*’) a 4 C em um compartimento umidificado. A detecção do anticorpo primário foi feita com anticorpo secundário biotinilado anti-coelho por 20 minutos e apos incubação em solução de streptavidina conjugada à peroxidase (streptavidin-HRP) a reação foi revelada com o uso do cromógeno DAB. Os cortes foram contra-corados com hematoxilina por 15 segundos, deixados secar a temperatura ambiente por 2 h e em seguida as lamínulas foram montadas com resina. As imagens foram capturadas em microscópio de luz (Diastar-Cambridge Instruments) na magnificação de 200X e parâmetros de captura da imagem (brilho, contraste, tempo de exposição) mantidos constantes.

Segundo otimização previamente realizada para imunohistoquimica de RANKL, o bloqueio da peroxidase endógena foi feito com incubação dos cortes com solução de peróxido de hidrogênio e metanol (1:1), 2 vezes por 15 minutos. As etapas de recuperação antigênica e bloqueio de proteínas inespecíficas não foram realizadas. Os cortes foram incubados com anticorpo policlonal de coelho anti-RANKL (Santa Cruz Biotechnology, sc-7628) diluído 1:100 em BSA 1% por 18 h (‘overnight’) a 4 C em um compartimento umidificado. O anticorpo secundário biotinilado anti-coelho foi incubado com os cortes durante 30 minutos. Os passos seguintes de revelação da reação e obtenção das imagens foram os mesmos descritos para o anticorpo NF-kB, utilizando o mesmo kit de reagentes (LSAB2, Dako Cytomation).

Extração de RNA total, reação de síntese de cDNA (transcrição reversa) e PCR em tempo real (qPCR)

Para o estudo in vitro um total de 3 x 10^5 células foram plaqueadas em placas de 6 poços e após 24 horas desinduzidas em meio de cultura contendo 0.3% de soro fetal bovino, e então estimulados com lipopolisacarídeo de *E. coli* por 24 horas, com e sem pré-tratamento com 5 µM ou 10 µM de curcumin. Células de outros poços foram tratadas com 10 µM do inibidor de p38 MAPK (SB203580) ou 10 µM do inibidor de NF-κB (Bay 11-7082). Nos poços correspondentes ao controle, as células foram tratadas com o mesmo volume de DMSO, veiculo usado na diluição destes componentes.

RNA total das células e dos tecidos gengivais foram extraídos com o reagente Trizol (Invitrogen Corp.) de acordo com as instruções do fabricante. A pureza e quantidade de RNA foram determinadas em espectrofotômetro pela leitura da absorbância a 260 nm e da relação entre as absorbâncias a 260 e 280 nm, respectivamente. 700 ng de RNA total foram utilizados para a síntese de cDNA utilizando random hexamers como primers e seguindo as instruções do fornecedor dos reagentes (High capacity cDNA synthesis kit, Applied Biosystems). A expressão dos genes inflamatórios selecionados foi determinada por RT-qPCR tempo real usando sondas e reagentes Taqman (TaqMan Gene Expression Assays, TaqMan Universal master mix, Applied Biosystems) em um sistema de PCR Tempo Real StepOne (Applied Biosystems). As reações foram feitas em placas de 96 poços com um volume final de 30 µL que inclui Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) para cada gene: tnf-alpha (tumor necrosis factor alpha), NM_013693; il-6 (interleukin 6), NM031168; Ptgs-2 (prostaglandin-endoperoxide synthase-2), NM011198; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), NM008084. As condições das reações de PCR foram: 50^{o}C por 2 min, 95^{o}C por 10 min, seguido por 40 ciclos a 95^{o}C por 15 segundos e 60^{o}C por 1

minuto. Para cada amostra, as análises da expressão gênica foram realizadas em duplicata. Para normalizar a quantidade de cDNA total presente em cada reação, a expressão de GAPDH, que não foi alterada pelas condições experimentais, foi usado como controle endógeno por ser um gene constitutivo. Para comparar os níveis de expressão entre as diferentes amostras, o nível de expressão relativa dos genes foi calculado usando o método comparativo ΔCT utilizando o software da termocicladora.

Determinação da expressão gênica a nível protéico (ELISA)

Sobrenadantes da cultura celular foram coletados dos mesmos poços que o RNA total foi coletado. Estes sobrenadantes foram aliquotados e estocados a -80 C. Amostras de proteína total foram extraídas dos tecidos periodontais removidos no momento do sacrifício. Para isto, foi utilizado o tampão de extração T-PER (Tissue Protein Extraction Reagent – Pierce Biotechnology, código de catálogo #78510) suplementado com um coquetel de inibidores de protease (Protein Stabilizing Cocktail – Santa Cruz Biotechnology, sc-29130). Este tampão foi adicionado aos tecidos, os quais foram macerados, centrifugados por 5 minutos a 13.000 RPM a 4 C, com posterior remoção do sobrenadante. As amostras de proteína contendo sobrenadante das culturas e as provenientes dos tecidos gengivais foram usadas para realização do ELISA para quantificação dos níveis de PGE2, IL-6 e TNF-ALPHA. Estas avaliações foram feitas de acordo com as instruções do fabricante dos kits ELISA (R&D Systems) e a concentração das proteínas-alvo foi normalizada para o conteúdo de proteína total determinado pelo método de Lowry (DC assay, Bio-Rad).

Ativação das vias de sinalização (Western Blot)

Para este experimento, 3 x 10^5 células por poço foram plaqueadas em cada placa de 6 poços, rotineiramente desinduzidas por 8 horas e em seguida tratadas com 5 ou 10

μM de curcumin por 30 minutos antes da estimulação com 1 μg/mL de LPS bacteriano. Experimentos controles foram feitos com a adição de 10 μM de SB203580, 10 μM do Bay 11-7082 ou o mesmo volume do veiculo DMSO. O estimulo com LPS foi mantido por 10, 30 e 60 minutos. No final de cada período experimental todo o lisado celular foi coletado raspando as células em tampão RIPA (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 ug/mL leupeptin, 1 mM PMSF) sobre gelo, seguido de incubação no gelo por 15 a 30 minutos, centrifugação à 12,000 RPM à 4 C por 10 minutos e transferência do sobrenadante para um novo tubo eppendorf e armazenamento à -80 C. As proteínas totais foram quantificada pelo método de Lowry (DC Assay, Bio-Rad). Para realização do Western Blot quantidades iguais de proteína total (40 μg) foram resolvidas por eletroforese. Para a eletroforese, as amostras de proteína foram adicionadas ao tampão de amostra contendo SDS 2% (3X SDS Sample Buffer Blue, Cell Signaling, #7722) e 41.7 mM de DTT e ajuste de um volume final igual para as amostras com o tampão de extração de proteínas. Esta homogenização foi feita em gelo, seguida de fervura para desnaturação protéica a 97 C por 5 minutos, posterior centrifugação de 30 segundos a 13,000 RPM (centrífuga 5415D – Eppendorf) e manutenção em gelo por 5 minutos, procedimentos também realizados para o padrão de peso molecular biotinilado (Cell Signaling).

Para o Western Blot, as amostras foram separadas em géis de poliacrilamida Tris-Cl a 10% (com o sistema Mini-Protean 3 cell – Bio-Rad Lab.), utilizando voltagem constante de 100 V por 90 minutos, seguidos de eletro-transferência (Mini Trans-blot Electrophoretic Transfer Cell – Bio Rad) para membranas de nitrocelulose (Bio-Rad Lab.) durante 1 hora a 300 mA constantes. Após o bloqueio em tampão Tris-NaCl (TBS) contendo 5% de leite desnatado liofilizado *(blocking buffer)* por 1 hora sob

agitação (TS-2000 VDRL *shaker* – Biomixer) à temperatura ambiente, as membranas receberam 3 lavagens de 5 minutos com tampão TBS-Tween (TBS-T) a 0,1% e foram incubadas sob agitação (Mini Rocker MR-1 – Biosan) *overnight* a 4°C com os anticorpos primários para as proteínas de interesse: phospho-p65, phospho-JNK, phospho-ERK e phospho-p38 MAPKinase (1:1000 em tampão de diluição de anticorpo primário (BSA 5% em PBS, pH 7.4) além de GAPDH (diluição 1:400, Santa Cruz Biotechnology) para confirmar que quantidades iguais de proteínas totais foram utilizadas. O anticorpo secundário utilizado (conjugado à *horseradish peroxidase)* foi diluído de acordo com as instruções do fabricante (Cell Signaling). Utilizou-se o anticorpo secundário anti-coelho conjugado à peroxidase (HRP) (diluição de 1:1000) em *blocking buffer.* Após remoção do anticorpo primário e 3 lavagens com TBS-T, a incubação com o secundário foi realizada por 1 hora à temperatura ambiente sob agitação (TS-2000 VDRL *shaker* – Biomixer). Em seguida, as membranas foram novamente lavadas e a detecção da presença das proteínas realizada por um sistema de quimioluminescência (LumiGlo, Cell Signaling). Imagens digitalizadas dos filmes radiográficos para Western Blot foram obtidas em um sistema de digital de fotodocumentação (ImageQuant 100 – GE Healthcare).

Microtomografia computadorizada (μCT)

As pecas destinadas a análise por microtomografia computadorizada foram fixadas em formol tamponado a 10% por 24 horas e então imersas e conservadas em etanol a 70% e mantidas a 4 C até o momento da análise, que foi realizada pela varredura de feixe de raios-X em um sistema de microtomografia computadorizada (GE HealthCare BioSciences). Cada *scan* (varredura) foi reconstruído em uma matriz de 18x18x18 μm e as imagens tridimensionais geradas para cada amostra. Estas imagens

foram rotacionadas em uma orientação-padrão e foi estabelecido um limite de contraste para distinguir tecidos mineralizados de não-mineralizados utilizando o software GEHC Microview (version Viz+2.0 build 0029 GE HealthCare BioSciences). Após determinação da região de interesse (ROI) correspondente as coroas dos dentes e a região do osso alveolar, foi utilizada uma caixa volumétrica padrão (250 x 200 x 200 pixels) para determinação da fração deste volume ocupado por tecido ósseo. Estas análises foram efetuadas no Orthopaedic Research Laboratory (Biomedical Engineering Dept, University of Michigan) segundo orientação do colaborador no exterior (Dr. Keith L Kirkwood, Department of Craniofacial Biology, Medical University of South Carolina-MUSC, Charleston, SC, USA).

Avaliação da perda óssea pela coloração da superfície radicular exposta com azul de metileno

As peças destinadas à quantificação do processo de reabsorção óssea alveolar através da técnica complementar de coloração com azul de metileno foram fixadas em formol tamponado a 10% por 24 horas e em seguida mantidas em solução de peróxido de hidrogênio por 24 horas e limpas para remoção do tecido mole remanescente. As peças foram então armazenadas em álcool 70% até o momento de serem coradas com azul de metileno. Este foi preparado com a adição de 0,7g do corante dissolvidos em 50 mL de etanol a 95% com posterior diluição de 2,5 mL dessa solução em 47,5 mL de água e manutenção das peças no corante durante 5 minutos. Em seguida, a face lingual das peças foi fotografada em estereomicroscópio (*Leica MZ6* - magnificação de 40X) e as mesmas analisadas por um examinador treinado e cego para os grupos experimentais. Assim, a região compreendida entre o início da raiz mesial do primeiro molar superior e a região entre a primeira e a segunda raiz do segundo molar superior foi considerada

para a mensuração da área de perda óssea, compreendida entre a junção cemento esmalte (JCE) e o osso alveolar. A área da lesão e extensão das reabsorções óssea e dentária foram analisadas histomorfometricamente utilizando programa ImageJ 1.34 (NIH Image).

DISCUSSÃO

Discussão

O efeito do curcumin sobre a modulação da doença periodontal experimentalmente induzida in vivo foi avaliada pela primeira vez neste trabalho. Nossos resultados demonstram que a administração de curcumin foi capaz de inibir significativamente a degradação do tecido conjuntivo e da matriz de colágeno nos tecidos gengivais onde a doença periodontal foi experimentalmente induzida, reduzindo o número de células inflamatórias e a produção de mediadores inflamatórios a níveis semelhantes aos do grupo controle. Este resultado pode ter sido um reflexo da ação modulatória do curcumin sobre a ativação de NF-κB.

Estes resultados estão de acordo com aqueles apresentados por outros autores que avaliaram o efeito do curcumin em modelos de doença inflamatória em animais, como colite, diabetes, artrite reumatóide, doença de Crohn [11, 24, 49, 65, 99, 119]. O potencial terapêutico do curcumin vem sendo explorado em diversos estudos clínicos no tratamento de vários tipos de câncer (próstata, mama, pulmão, linfoma) e em outras condições como artrite, mal de Alzheimer, diabetes e depressão [35, 38, 53, 111, 115, 116, 121]. Nestes estudos in vivo, curcumin inibiu a expressão dos mediadores inflamatórios associados a estas patologias, como IL-1beta, MMPs, COX-2, IL-6 e TNF-alfa, além de inibir vias de sinalização e fatores de transcrição geralmente ativados em processos inflamatórios, como NF-κB, AP-1, STAT-3 e as MAPKinases. Curcumin também pode ter relevância como fitoterápico na reparação de feridas cutâneas em animais diabéticos, favorecendo a neovascularização, reepitelização e na migração de células como fibroblastos e macrófagos [119]. Similarmente aos nossos resultados, no estudo de Sidhu et al. [119] a administração tópica e sistêmica (gavagem oral) de curcumin provocou um aumento no conteúdo de colágeno em feridas experimentais [119]. Em outro estudo, a administração oral de 100mg/kg/peso corporal de curcumin, a maior dose utilizada no nosso trabalho, 2 horas previamente a indução de colite, também foi capaz de reverter

as alterações histológicas associadas, incluindo aumento no número de neutrófilos e eosinófilos na camada epitelial, necrose hemorrágica e ulceração [82]. Resultado similar também foi encontrado no estudo de Swarnakar et al. [125], em que a administração (oral e intraperitoneal) de curcumin em ratos bloqueou a ulceração gástrica de maneira dose-dependente. Curcumin acelerou o processo de cicatrização e reduziu a severidade da úlcera gástrica atenuando a atividade de MMP-9 e MMP-2 [83]. Estes resultados sugerem que curcumin atua não apenas inibindo a resposta inflamatória, mas também favorecendo o processo de reparo tecidual.

A inibição de mediadores inflamatórios como IL-6, TNF-alfa e IFN-gama também foi relatada em ratos tratados com curcumin administrado via oral em doses superiores às utilizadas no nosso estudo (200 mg/kg e 400 mg/kg). Além do fato de doses elevadas serem possíveis e bem toleradas pelos animais experimentais, curcumin teve efeito hepatoprotetor em modelo animal de injúria hepática induzida por tetracloreto de carbono (CCL_4) ao reduzir a atividade das enzimas aspartato aminotransferase sérica, alanina aminotransferase, e fosfatase alcalina, ao mesmo tempo em que melhorou a arquitetura histológica do fígado [43]. De maneira semelhante, curcumin também diminuiu a inflamação associada a dois modelos experimentais de pancreatite através de uma forte redução na ativação de NF-κB e AP-1 e inibição da produção de IL-6, TNF-alfa e iNOS no pâncreas [49]. Em ambos os modelos o efeito inibitório de curcumin sobre os mediadores inflamatórios resultaram em redução da severidade da pancreatite, avaliada histologicamente, pelo número de neutrófilos infiltrados e pela diminuição do nível de tripsina pancreática [49]. Estes resultados não apenas confirmam a ação anti-inflamatória do curcumin sobre os níveis de citocinas pró-inflamatórias como também demonstram a ausência de toxicidade de doses superiores àquelas utilizadas em nosso estudo.

Com relação ao efeito do curcumin em modelos clínicos, os resultados também parecem promissores. Deodhar e colaboradores [35] randomizaram 18 pacientes com artrite reumatóide para receber o tratamento com curcumin (1200 mg/dia) ou fenilbutazona (300mg/dia) por 2 semanas. Os pacientes que receberam curcumin tiveram uma melhora nos sintomas da artrite reumatóide tão substancial quanto aqueles que receberam fenilbutazona, e a administração do curcumin não causou nenhum efeito colateral discernível. Em outro estudo, Holt et al. [53] reportaram o uso do curcumin no tratamento de doença inflamatória intestinal. Cinco pacientes com doença de Crohn foram tratados com curcumin 360 mg três vezes ao dia por 1 mês, seguido por 360 mg quatro vezes ao dia diariamente, durante os 2 meses seguintes. Quatro dos cinco pacientes com doença de Crohn tiveram os sintomas atenuados, o que foi evidenciado pela melhora no índice de atividade da doença (CDAI).

Devido a sua habilidade em eliminar radicais livres e inibir a inflamação, curcumin tem sido investigado também na quimioprevenção de câncer e supressão do crescimento tumoral [10, 31, 57, 59, 60, 106]. Curcumin tem mostrado prevenir o desenvolvimento de cânceres do estomago, duodeno, língua, cólon e glândulas mamárias em modelos de carcinogênese química em ratos e camundongos [7, 26, 37, 89]. No entanto, em um estudo clinico fase I avaliando o efeito do curcumin em cânceres avançados, Kutan et al. [76] relataram que 10% dos 62 pacientes que fizeram uso do curcumin como tratamento tópico para câncer oral e leucoplasia mostraram redução no tamanho da lesão [76].

O resultado destes e de diversos outros estudos demostrando o efeito anti-inflamatório do curcumin no tratamento de doenças de natureza inflamatória tanto em modelos animais como em estudos clínicos consolidam os resultados apresentados neste trabalho, em que a administração de curcumin in vivo reduziu significativamente a

expressão dos mediadores pró-inflamatórios IL-6, TNF-alfa e PGE-2 associadas a dois modelos de doença periodontal experimentalmente induzida. Estes efeitos são, ao menos em parte, devidos à modulação negativa da ativação do fator de transcrição NF-κB, além da inibição do processo inflamatório observada histologicamente.

Curcumin é considerado uma substância pleiotrópica pela sua capacidade de interagir com diversos alvos moleculares envolvidos na inflamação. Ele modula a resposta inflamatória reduzindo a atividade de mediadores pró-inflamatórios como COX-2, lipoxigenase e iNOS, inibindo a produção de citocinas inflamatórias como TNF-alfa, IL-6, IL-1beta, IL-8, IL-12 e modulando a ativação de vias de sinalização relacionadas à transdução de sinais intracelulares envolvidas na expressão destes mediadores inflamatórios induzidas por diversos estímulos externos, bem como das vias de sinalização utilizadas na transdução de sinais por estes mediadores inflamatórios [46].

A inibição de muitos destes mediadores inflamatórios é acompanhada pela supressão principalmente do fator de transcrição NF-κB, envolvido na regulação da inflamação, proliferação e transformação celular e tumorigêneses [124] e considerado um dos principais ‘alvos moleculares’ do curcumin. Curcumin parece suprimir a ativação de NF-κB e a expressão de genes pró-inflamatórios através do bloqueio da fosforilação do fator Inhibitor of kappa B kinase (IκB). A supressão da ativação de NF-κB, ativada por sinais externos para a indução de mediadores inflamatórios e também ativada pelos próprios mediadores inflamatórios, subsequentemente modula os processos imunes e neoplásicos [64, 124].

Por exemplo, o estímulo de condrócitos articulares com TNF-alfa e IL-1beta resulta em ativação de NF- κB. O tratamento das células com curcumin por mais de 72 horas suprimiu a ativação de NF-κB induzida pelas citocinas através da inibição da fosforilação e degradação de IκBα, prevenindo a fosforilação e translocação nuclear de

p65, reduzindo desta forma a expressão de genes-alvo de NF-κB, incluindo COX-2 e MMP-9 [113]. Estes resultados sugerem que a inibição dos mediadores inflamatórios que reportamos nos estudos in vivo, pode ter sido, ao menos em parte, mediada pela inibição da ativação de NF-κB pelo curcumin.

O efeito anti-inflamatório do curcumin sobre outros tipos celulares específicos relacionadas à resposta imune inata e adaptativa como macrófagos, linfócitos T e B, neutrófilos, células endoteliais e fibroblastos também têm sido estudado. Brouet, I. [22] mostrou que o tratamento de macrófagos com curcumin em diferentes concentrações inibiu a expressão de NOS induzida por estímulo com LPS bacteriano (*E.coli* 1 ug/mL) e interferon-gama [22]. Esta inibição alcançou maior expressão quando curcumin foi adicionado ao meio de cultura ao mesmo tempo em que as células foram estimuladas. Este resultado é semelhante ao que reportamos no experimento in vitro, em que o tratamento de macrófagos murinos com curcumin (5 e 10µM) foi capaz de reduzir os níveis de PGE-2, IL-6 e TNF-alfa estimulados com a mesma concentração de LPS utilizada no trabalho de Brouet. Este efeito inibitório do curcumin sobre a ativação de NF-κB estimulada por LPS também foi relatada recentemente por Nishida et al. [92]. Monoacetilcurcumin, um análogo de curcumin, inibiu fortemente a ativação de NF-κB e a produção de TNF-alfa em macrófagos estimulados por LPS [92]. Chen, D. et al. [25] obteve resultado bastante semelhante quando estimulou macrófagos com LPS de *Porphyromonas. gingivalis.* A expressão protéica de TNF-alfa e IL-1beta induzidas pelo LPS foram inibidas de maneira dose-dependente pelo curcumin adicionado 2 horas previamente ao estimulo [25].

Além de NF-kB, existem evidências de que o curcumin pode modular a atividade das MAPKinases, as quais também tem papel relevante na resposta imune-inflamatória. Células endoteliais humanas estimuladas com 10 ng/mL de TNF-alfa e

tratadas com curcumin apresentaram não somente bloqueio da atividade de NF-κB, mas também de p38, JNK e STAT-3 [71]. Similarmente, curcumin inibiu a produção de IL-6, IL-1beta e TNF-alfa e a ativação das MAPKinases (p38, JNK e ERK) em células epiteliais humanas não tumorais (HaCat) estimuladas com TNF-alfa [28]. Estes relatos estão de acordo com os resultados de nosso experimento in vitro, em que o tratamento de macrófagos murinos com curcumin (5 e 10 μM) inibiu a ativação de JNK, ERK, e p38 MAPKinases induzida por LPS bacteriano.

Entretanto, em contraste com os resultados de nosso estudo in vitro, a administração oral de curcumin 30 mg/kg e 100 mg/kg in vivo não afetou a atividade de p38 MAPKinase nos tecidos gengivais de ratos com indução da doença periodontal segundo o modelo de ligadura ou de LPS. Este resultado também difere do relatado no estudo de Camacho-Barquero et al. [24] em que ratos com colite induzida tratados com curcumin (50-100 mg/kg) durante 15 dias diariamente, tiveram uma redução na ativação de p38 MAPK [24]. Alguns estudos, entretanto, têm mostrado resultados contraditórios ao avaliar a modulação de p38 pelo curcumin. Queratinócitos humanos estimulados por radiação ultra-violeta tipo B (UVB) apresentaram ativação de p38 MAPK mais intensa quando tratados simultaneamente com 20 uM de curcumin do que quando apenas expostos ao UVB [9]. Neutrófilos periféricos obtidos de humanos saudáveis tiveram um marcado aumento na ativação de p38 após exposição das células ao curcumin [56]. Segundo os autores, o aumento na atividade de p38 MAPK pode estar relacionado ao efeito pró-apoptótico, indicado pela ativação de caspase-3, do curcumin sobre os neutrófilos humanos [56].

A indução de apoptose representa outro efeito biológico importante do curcumin, principalmente no processo de inibição tumoral. Os mecanismos responsáveis por este processo parecem ser variados, incluindo efeitos sobre a

estabilidade de p53, a liberação do citocromo c e a geração de espécies de oxigênio reativo (ROS) [3, 129]. A indução de apoptose pelo curcumin em células epiteliais da mama envolveu a redução da expressão de Bcl-2 e geração de ROS [70]. Em células AK-5 tumorais a atividade anti-tumoral do curcumin mostrou-se estar relacionada a indução de apoptose através da ativação de caspase-3 e da geração de ROS [15, 69]. Em geral, estudos investigando os efeitos apoptóticos do curcumin concluem que este processo pode ser induzido em células cancerígenas através das vias intrínseca (caspases) e metabólica (mitocôndria/citocromo c), sendo que a via preferencialmente utilizada pelo curcumin pode depender do tipo celular, estágio de diferenciação celular ou concentração do curcumin [109].

Na doença periodontal em humanos, a ocorrência de apoptose, verificada pela marcação de TUNEL, foi verificada em biópsias de pacientes com periodontite, porém não foram observadas nas biópsias de pacientes saudáveis. O tipo celular mais afetado pela apoptose nos tecidos gengivais inflamados foram neutrófilos [44]. De forma semelhante a presença de caspase-3 ativada foi detectada em tecidos gengivais humanos inflamados, enquanto biópsias de tecidos gengivais saudáveis foram negativas para presença de caspase-3 ativada. Finalmente, a expressão de Bcl-2 foi detectada tanto nos tecidos saudáveis quanto nos tecidos com periodontite. Entretanto, um maior número de células positivas para Bcl-2 foram observadas no infiltrado inflamatório de pacientes com doença periodontal [44]. Este aumento da expressão de Bcl-2 associado à inflamação foi verificado também em amostras de tecido gengival de pacientes com periodontite agressiva. O aumento da expressão de Bcl-2 pode indicar uma inibição da apoptose, a qual pode prolongar a presença de células inflamatórias, em maior quantidade nos tecidos inflamados, resultando na persistência da inflamação e maior destruição periodontal [23].

Nossos resultados mostram que a expressão protéica de Bcl-2 nos tecidos gengivais periodontalmente comprometidos de animais tratados com curcumin foi significativamente reduzida em relação ao grupo não tratado, sugerindo que a administração de curcumin aumentou a apoptose celular nos tecidos gengivais. Entretanto, em contraste com os estudos relatando aumento da expressão de Bcl-2 na doença periodontal, em ambos modelos de doença periodontal em ratos não observamos aumento marcante da expressão de Bcl-2 com a inflamação. Como não avaliamos a expressão e atividade de outros mediadores do processo apoptótico que poderiam estar atuando nos tecidos gengivais destes animais, não podemos conclusivamente afirmar que a administração de curcumin modulou a apoptose nos tecidos gengivais na presença de doença periodontal induzida experimentalmente.

Além disso, estes resultados são derivados da avaliação do extrato protéico total dos tecidos gengivais por western-blot. Ainda que o mecanismo associado à modulação de Bcl-2 pelo curcumin não tenha sido explorado neste trabalho, é interessante especular que a apoptose induzida pelo curcumin in vivo pode afetar preferencialmente células inflamatórias, uma vez que a avaliação histológica demonstra que a vascularização e reparação não foram afetadas negativamente pelo curcumin e que resultados de trabalhos anteriores observaram que a população de células apoptóticas encontradas nos tecidos gengivais de pacientes com periodontite são predominantemente neutrófilos. Esta hipótese de indução de apoptose preferencialmente em células inflamatórias poderia também estar relacionada à redução da produção de mediadores inflamatórios nos tecidos dos animais tratados com curcumin: menor número de células inflamatórias viáveis nos tecidos proporcionaria redução da produção de citocinas inflamatórias. Estudos complementares serão realizados para tentar obter maior ‘*insight*’ no papel da apoptose associada à administração de curcumin in vivo, com a avaliação da localização

dos níveis de Bcl-2 e caspase-3 nos tecidos gengivais por meio de imunofluorescência e determinação dos tipos celulares (células inflamatórias x fibroblastos) mais afetados por esta apoptose.

Com relação aos efeitos do curcumin sobre células do sistema imune, existem evidências sugerindo que curcumin pode modular a proliferação e a ativação das células T, células dendríticas, células NK e células B além de macrófagos e neutrófilos (para revisão consulte: [63]. A proliferação de linfócitos T derivados do baço humano estimulados por concavalina A (Com A), fitohemaglutinina (PHA) e 13-acetato-12-myristate-forbol (PMA) foi inibida pelo curcumin. No mesmo estudo, IL-2 induziu a proliferação de linfócitos e curcumin suprimiu a síntese de IL-2. Esta supressão da produção de IL-2 foi correlacionada com a supressão da ativação de NF-κB pelos autores. Estes resultados sugerem que curcumin exibe efeitos supressivos em células T mediados pela da regulação de IL-2 [135].

Alguns poucos estudos têm relatado também o efeito do curcumin na resposta imune inata, por meio da modulação da sinalização dos receptores semelhantes à Toll [47, 82, 136]. Existem diversas evidências demonstrando que vários polifenóis inibem a ativação de NF-κB iniciada pelo LPS bacteriano, mas na maioria dos casos o alvo molecular foi claramente estabelecido [17, 30]. Resultados mostrando a capacidade do curcumin em inibir os receptores Toll são de grande relevância farmacológica uma vez que evidenciam de forma direta seu potencial na supressão de reação inflamatória causadas por infecções bacterianas ou virais [47, 82, 136]. No presente trabalho, relatamos que curcumin inibiu a expressão de mediadores pró-inflamatórios tanto no estudo in vivo, em modelo de doença periodontal induzida por LPS, quanto no estudo in vitro, no qual a expressão destes mediadores inflamatórios foi induzida em macrófagos através do estímulo de LPS. Estes resultados sugerem que o curcumin pode modular a resposta

imune inata, no entanto estes dados deverão ser expandidos por estudos utilizando inibidores de síntese protéica para confirmação de que a inibição da expressão de mediadores inflamatórios induzidos por LPS pelo curcumin ocorre de forma direta e não indireta. O nível de expressão dos receptores TLR2 e TLR4 também será avaliado para verificar um Possível papel do curcumin na 'dessensibilização' dos macrófagos. De qualquer forma, os resultados sugerem um papel potencial para o curcumin no tratamento de inflamações crônicas causadas por infecções bacterianas, como é o caso não apenas da doença periodontal, mas também de cânceres gástricos que se iniciam por inflamações gástricas crônicas causadas por bactérias gastrointestinais [87].

Embora os resultados deste estudo tenham confirmado alguns dos efeitos biológicos atribuídos ao curcumin e demonstrados em inúmeros trabalhos pré-clinicos e clínicos, como por exemplo, sua ação anti-inflamatória com inibição da expressão de genes pró-inflamatórios induzidos in vitro pelo estimulo com LPS e in vivo em dois modelos experimentais de doença periodontal; redução do processo inflamatório e inibição da degradação da matriz colágena dos tecidos periodontais; redução da ativação do fator de transcrição NF-κB nos estudos in vivo, e das MAPKinases no estudo in vitro; curcumin não protegeu o tecido ósseo periodontal da reabsorção óssea induzida pelos modelos de doença periodontal experimental utilizados.

Análise da microtomografia óssea realizada na região de interesse ao redor dos primeiros molares inferiores de todos os grupos experimentais mostra que a colocação da ligadura provocou uma redução na fração de volume ósseo, e que o tratamento com curcumin não preveniu a reabsorção óssea. Resultado semelhante foi obtido com os molares superiores destinados à quantificação do processo de reabsorção óssea alveolar através da técnica complementar de coloração com azul de metileno. A área de

reabsorção óssea nos grupos tratados ou não com curcumin não foi estatisticamente diferente.

Experimentos avaliando o papel do curcumin na reabsorção óssea têm mostrado resultados promissores na prevenção da atividade osteolítica. Células mononucleares derivadas do sangue periférico de voluntários saudáveis foram tratadas com curcumin (1 µM e 10 µM), o qual foi capaz de inibir de forma dose-dependente tanto a diferenciação (osteoclastogênese) induzida por RANKL quanto a atividade dos osteoclastos diferenciados, sugerindo potencial do curcumin no tratamento de doenças ósseas [132]. No estudo de Bharti, A.C. et al. [14] o tratamento de macrófagos murinos Raw 264.7 com RANKL ativou NF-κB e a pré-exposição destas células ao curcumin suprimiu completamente esta ativação. RANKL induziu a diferenciação dos macrófagos em osteoclastos tanto na ausência quanto na presença do curcumin, porém o número de osteoclastos formados foi reduzido com o aumento da concentração do curcumin. Resultados deste estudo mostram também que RANKL induziu a formação de lacunas ósseas e que esta formação foi significantemente inibida pelo curcumin [14].

O efeito de curcumin sobre a atividade osteoclástica em ratos com diabetes induzida por estreptozotocina foi avaliado por Hie, M. et al. [52]. A dieta suplementar com curcumin reverteu o aumento nos níveis de atividade enzimática e de RNA mensageiro da fosfatase ácida resistente ao tartarato (TRAP) e catepsina K à níveis similares aos dos animais-controle não-diabéticos. A análise histoquímica mostrou que o aumento nas células TRAP-positivas no fêmur distal de ratos diabéticos foi reduzido ao nível controle pela suplementação dietética com curcumin, entretanto, os níveis de cálcio e atividade da fosfatase alcalina não foram diferentes entre o grupo diabético tratado ou não com curcumin. Além disso, não houve diferença entre os níveis de mRNA de RANKL nas amostras do fêmur distal entre os grupos de animais controle, diabéticos e

diabéticos tratados com curcumin [52]. Estes resultados estão de acordo com nossas observações in vivo, e sugerem um efeito direto do curcumin na osteoclastogênese, atuando diretamente em células precursoras de osteoclastos, porém de forma independente da expressão de RANKL por linfócitos T e células do estroma (fibroblastos e osteoblastos).

De fato, a ausência de modulação da expressão de RANKL pode representar uma possível explicação para ausência do efeito protetor do curcumin sobre a reabsorção óssea apesar de seu potente efeito anti-inflamatório. De acordo com nossos resultados, o tratamento com curcumin não reduziu expressão de RANKL quando comparado aos animais não tratados. É importante destacar que não encontramos na literatura estudos in vivo relatando a modulação da expressão de RANKL por curcumin, assim de forma especulativa propomos duas hipóteses para a ausência de efeito do curcumin na reabsorção óssea in vivo: 1) a complexidade da resposta imune associada às doenças periodontais, com o estabelecimento de uma rede de citocinas que podem incluir mediadores não modulados pelo curcumin e capazes de induzir a expressão de RANKL e 2) a ausência de efeito do curcumin na expressão gênica de RANKL em alguns tipos celulares específicos (por ex., linfócitos T, fibroblastos ou osteoblastos). Também é possível a associação destas possibilidades. Estudos complementares deverão avaliar os efeitos do curcumin na expressão gênica e ativação do promotor de RANKL induzida por ligantes de receptores do tipo Toll e por citocinas inflamatórias.

Alguns estudos clínicos avaliando a expressão de RANKL nos tecidos gengivais e no fluido gengival crevicular de indivíduos com periodontite, relatam aumento na expressão de RANKL em indivíduos com periodontite crônica comparados com indivíduos saudáveis [68, 134]. Dessa forma, considerando a relação entre a doença periodontal e a presença de níveis elevados de RANKL e seu papel na indução da

osteoclastogênese, a ausência de modulação da expressão de RANKL nos animais tratados com curcumin pode explicar o fato da reabsorção óssea não ter sido estatisticamente diferente entre os grupos periodontalmente doentes, tratados ou não com curcumin.

De maneira geral, embora os questionamentos propostos neste trabalho tenham sido esclarecidos, um melhor entendimento sobre os mecanismos pelos quais o curcumin realiza seus efeitos anti-inflamatórios durante o desenvolvimento da doença periodontal se faz necessário. Avaliação sistêmica da expressão gênica dos mediadores pró-inflamatórios avaliados, e até mesmo a caracterização do perfil de resposta Th1 ou Th2 na presença ou ausência de tratamento com curcumin, poderia nos dar uma resposta mais abrangente sobre o efeito do curcumin diante do processo de inflamação crônica. Da mesma forma, a realização de experimentos que explorem os mecanismos pelos quais o curcumin foi capaz de reduzir de maneira tão surpreendente o processo inflamatório observado histologicamente, analisando, por exemplo, seu efeito sobre a quimiotaxia e migração de células inflamatórias, tais como os neutrófilos, poderia nos fornecer informações interessantes e esclarecedoras.

Ainda com relação ao efeito do curcumin sobre o tecido conjuntivo, observamos a necessidade da realização de experimentos que avaliem o processo de proliferação e produção de fibras colágenas pelos fibroblastos gengivais, de maneira a entender melhor o aumento da presença de fibras colágenas induzido nos animais tratados com curcumin, observado tanto neste estudo como em outros modelos de doença crônica. Avaliação dos níveis de expressão das metaloproteinases da matriz e seu estado de ativação também pode ajudar a elucidar o mecanismo pelo qual curcumin foi capaz de inibir a degradação da matriz extracelular não-mineralizada.

Análise dos efeitos do curcumin administrados por vias diferentes da utilizada, seja como suplemento alimentar ou aplicado topicamente sobre os tecidos gengivais, também são propostas que podem fazer parte de estudos futuros, assim como o delineamento de períodos experimentais mais longos que possam talvez, mostrar algum efeito preventivo do curcumin sobre a reabsorção óssea, o que não foi observado no período de 15 dias utilizado em nosso trabalho. Também como proposta para trabalhos futuros, os efeitos do curcumin podem ser investigados com a administração do fitoterápico após remoção do estimulo patológico, obtendo desta forma informações a respeito dos seus possíveis efeitos no processo de reparação tecidual.

Frente às limitações do nosso trabalho podemos sugerir que o curcumin apresenta algum potencial no tratamento de doenças crônicas como a doença periodontal, entretanto, diante dos resultados promissores encontrados, o delineamento de experimentos que possam obter o melhor entendimento dos mecanismos inerentes a estes efeitos biológicos observados torna-se não apenas interessante como necessário.

CONCLUSÃO

Conclusão:

Curcumin tem potencial terapêutico no tratamento de doenças crônicas associadas com a sinalização imune inata, como a doença periodontal e infecções do trato intestinal. Na doença periodontal, benefícios como a redução no número de células inflamatórias e aumento de fibras colágenas podem ser obtidos com administração do curcumin. A inibição do fator de transcrição NF-κB e a modulação de genes pró-inflamatórios pelo curcumin, observados tanto no estudo in vitro quanto in vivo, podem ser os principais fatores responsáveis por estes efeitos anti-inflamatórios.

Por outro lado, curcumin não teve um efeito preventivo sobre a reabsorção óssea induzida. A ausência de modulação de RANKL pelo curcumin, e/ou de outros mediadores que possam induzir a expressão de RANKL, pode ser uma explicação para este fato.

Estudos avaliando seu efeito sobre a reabsorção óssea em períodos experimentais mais longos ou usando formas de administração e dosagens diferentes das utilizadas neste estudo podem trazer resultados diferentes destes encontrados.

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ANEXO

Outros Resultados

Curcumin inibe a expressão de Bcl-2 em ambos os modelos experimentais de doença periodontal

Considerando os efeitos pró-apoptóticos do curcumin relatados na literatura, avaliamos seu efeito sobre a expressão protéica de Bcl-2 nos tecidos gengivais saudáveis e periodontalmente doentes, dos animais tratados com curcumin ou veiculo nos dois modelos de indução da doença através da técnica de Western-blot (Figura 2). A indução de doença periodontal provocou não alterou significativamente a expressão de Bcl-2, indicando que a inflamação associada não foi à apoptose celular pela via metabólica. Curcumin reduziu a expressão de Bcl-2 nos tecidos gengivais em ambos os modelos experimentais, sugerindo que a administração de curcumin pode ter induzido apoptose. É importante destacar que estes resultados representam os níveis de Bcl-2 no extrato de proteínas totais obtido dos tecidos gengivais, incluindo todos os tipos celulares presentes, em especial células inflamatórias (neutrófilos, linfócitos B e T e macrófagos), bem como células residentes (fibroblastos, células endoteliais, células epiteliais), não sendo possível avaliar se a modulação da expressão de Bcl-2 e indução de apoptose ocorreu em alguns destes tipos celulares preferencialmente. Além disso, avaliamos a expressão de Bcl-2 com base em relatos da literatura que indicam a modulação da expressão desta proteína por curcumin, e não avaliamos a apoptose pela via que inclui a ativação das caspases. Assim, é possível que estejamos subestimando (caso a via de caspases também tenha sido ativada) ou superestimando (caso a ativação das caspases tenha sido inibida) a ocorrência de apoptose nos tecidos gengivais dos animais tratados com curcumin.

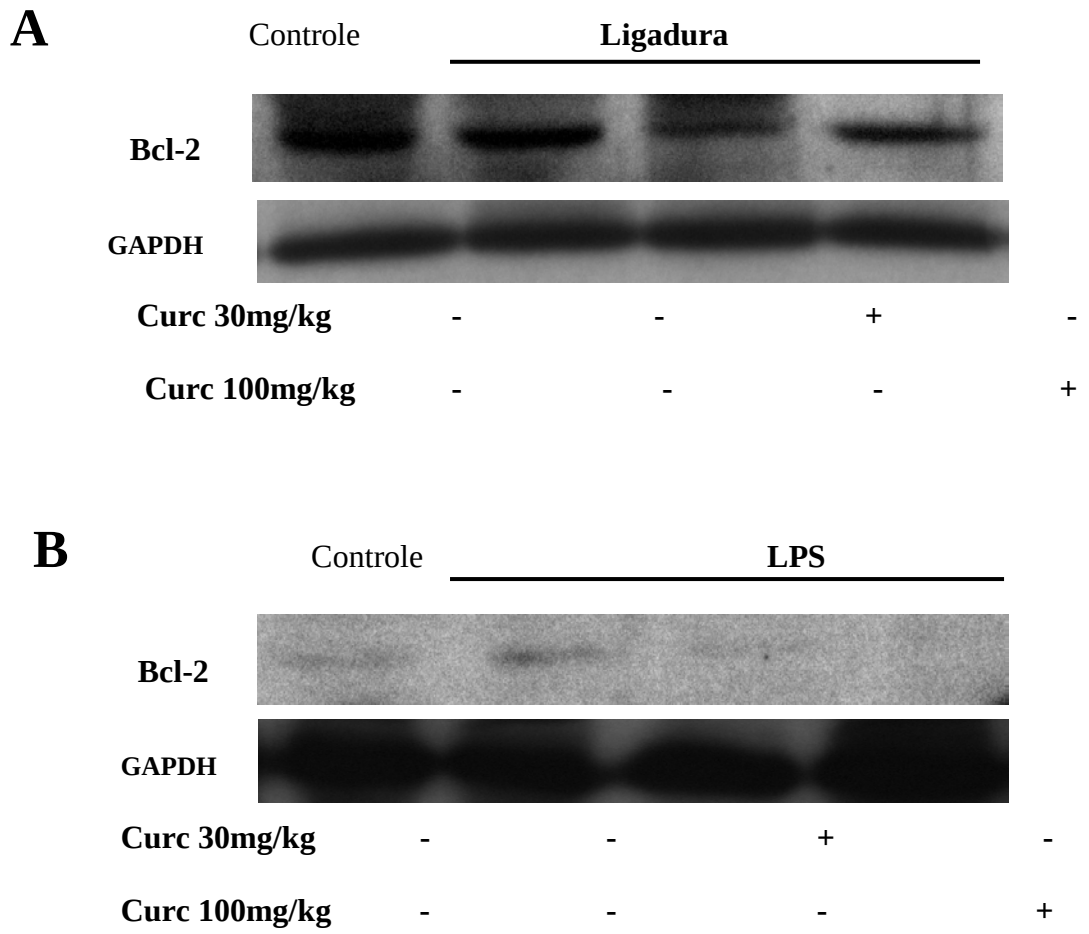


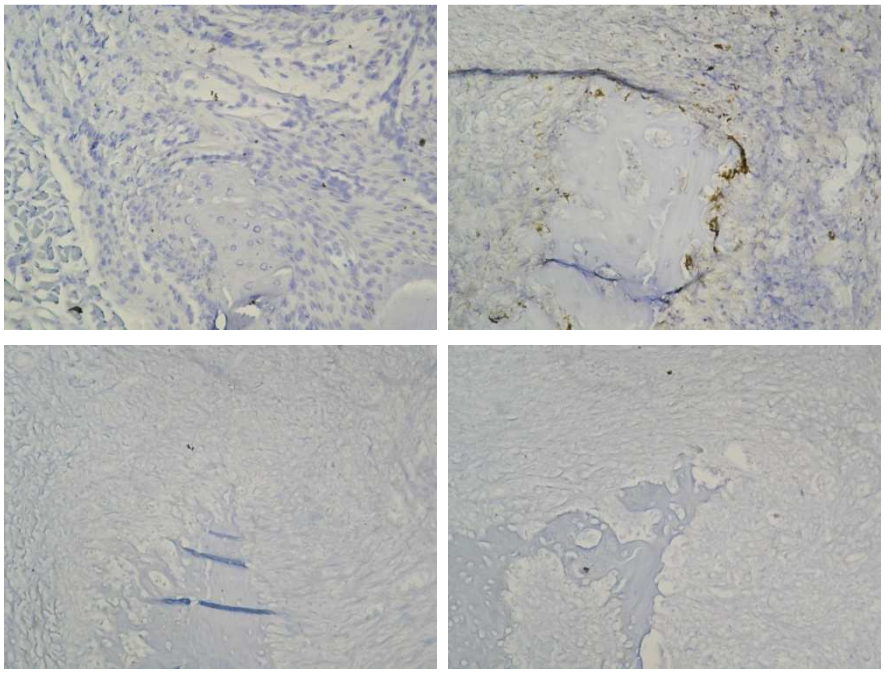
FIGURA 2: Inibição da expressão de Bcl-2 nos tecidos gengivais nos dois modelos experimentais de doença periodontal por curcumin. Proteínas totais foram extraídas dos tecidos gengivais saudáveis ou periodontalmente doentes de animais tratados ou não com curcumin e separadas em gel descontínuo de poliacrilamida por eletroforese para posterior detecção da proteína anti-apoptótica Bcl-2. O painel A mostra que a indução da doença periodontal pelo modelo de ligadura, não alterou significativamente a expressão de Bcl-2 em relação aos animais controle, entretanto, a administração do curcumin reduziu a expressão de Bcl-2. Esta modulação foi similar nos dois modelos experimentais (A, ligadura; B, LPS). Notar que em B (modelo de LPS), foi necessário carregar uma maior quantidade de proteína total para possibilitar a detecção de Bcl-2. A expressão de GAPDH foi utilizada como gene constitutivo não alterado pela indução de doença periodontal e pelo tratamento com curcumin para normalização dos

resultados. As figuras são representativas dos resultados obtidos em experimentos realizados utilizando amostras protéicas obtidas de um mínimo de três animais por grupo experimental.

Curcumin inibe a ativação de NF-κB no modelo de ligadura, mas não modula os níveis de RANKL nos tecidos periodontais

Após verificar a inibição da ativação de NF-kB por meio de western-blot de extratos protéicos preparados dos tecidos gengivais, avaliamos a localização da ativação de NF-κB por imunohistoquímica (Figura 3, painel A). Para avaliar os possíveis efeitos do curcumin na reabsorção óssea, determinamos, também por imunohistoquímica, a expressão de RANKL nos cortes histológicos incluindo primeiros molares inferiores de todos os grupos experimentais (Figura 3, painel B). Em suporte aos dados obtidos por meio de western-blot, a ativação de NF-κB estimulada pela colocação da ligadura foi marcadamente inibida, tanto no tecido conjuntivo supracrestal quanto nas células adjacentes à crista óssea alveolar, pelas duas doses de curcumin administradas, entretanto, nenhuma das doses afetou de forma marcante a expressão de RANKL nos tecidos periodontais. Interessante notar que a expressão de RANKL nos tecidos periodontais teve um padrão mais difuso, incluindo tanto o tecido conjuntivo supra-crestal quando a porção coronal do ligamento periodontal e a região adjacente à crista óssea.

A


Controle
Ligadura
NF-κB
NF-κB
Curc 30mg/kg +Ligadura
Curc 100mg/kg + Ligadura

B

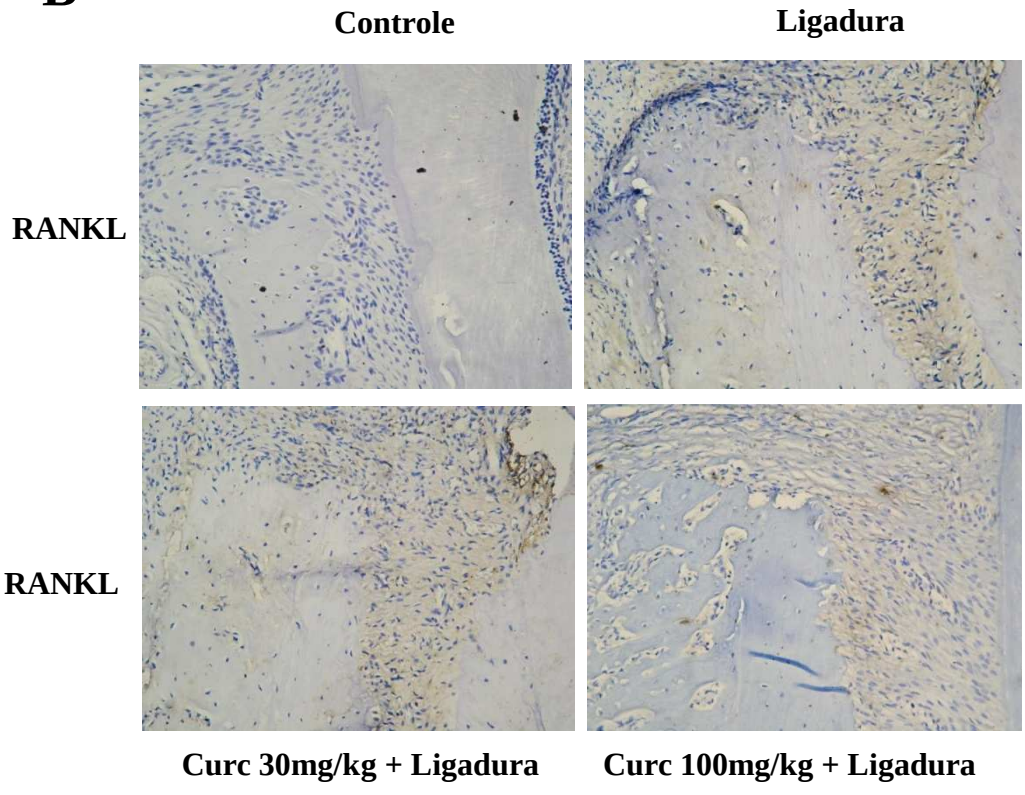

Controle
Ligadura
RANKL
RANKL
Curc 30mg/kg + Ligadura
Curc 100mg/kg + Ligadura

FIGURA 3: Imagens representativas dos resultados da imunohistoquímica para NF-κB (A) e RANKL (B). Cortes semi-seriados de 5 uM foram obtidos das peças incluídas em parafina e submetidos a procedimento-padrão de imunohistoquímica utilizando anticorpos primários policlonais de coelho para p50 (NF-kB) e RANKL. No painel A nota-se a presença de p50 indicando a ativação de NF-κB nos animais submetidos à indução de doença periodontal, a qual foi reduzida de forma marcante pelo tratamento com curcumin. No painel B observamos que a indução da doença periodontal estimulou a expressão de RANKL principalmente na região situada entre a crista óssea e o dente. Neste caso, o tratamento com curcumin não inibiu a expressão de RANKL estimulada pela ligadura. As imagens são representativas dos resultados avaliados em três cortes histológicos de um mínimo de três animais por grupo experimental.

A indução da doença periodontal e a administração do curcumin não alteram os níveis séricos de cálcio e fósforo

Considerando os efeitos já descritos em outros trabalhos, de modulação dos marcadores ósseos pelo curcumin, e a fim de se determinar se a administração do curcumin poderia alterar os parâmetros bioquímicos de formação óssea no soro de ratos submetidos ou não a indução da doença periodontal, os níveis de cálcio e fósforo foram avaliados no soro (Figura 4). Uma alíquota do sangue de todos os animais dos grupos experimentais foi retirado por punção cardíaca nos animais anestesiados imediatamente antes do sacrifício por aprofundamento da anestesia. Os resultados mostram que a indução da doença periodontal e a reabsorção óssea resultante não alteraram significativamente os níveis de Cálcio e Fósforo sérico, que também se mantiveram constantes com a administração do curcumin.

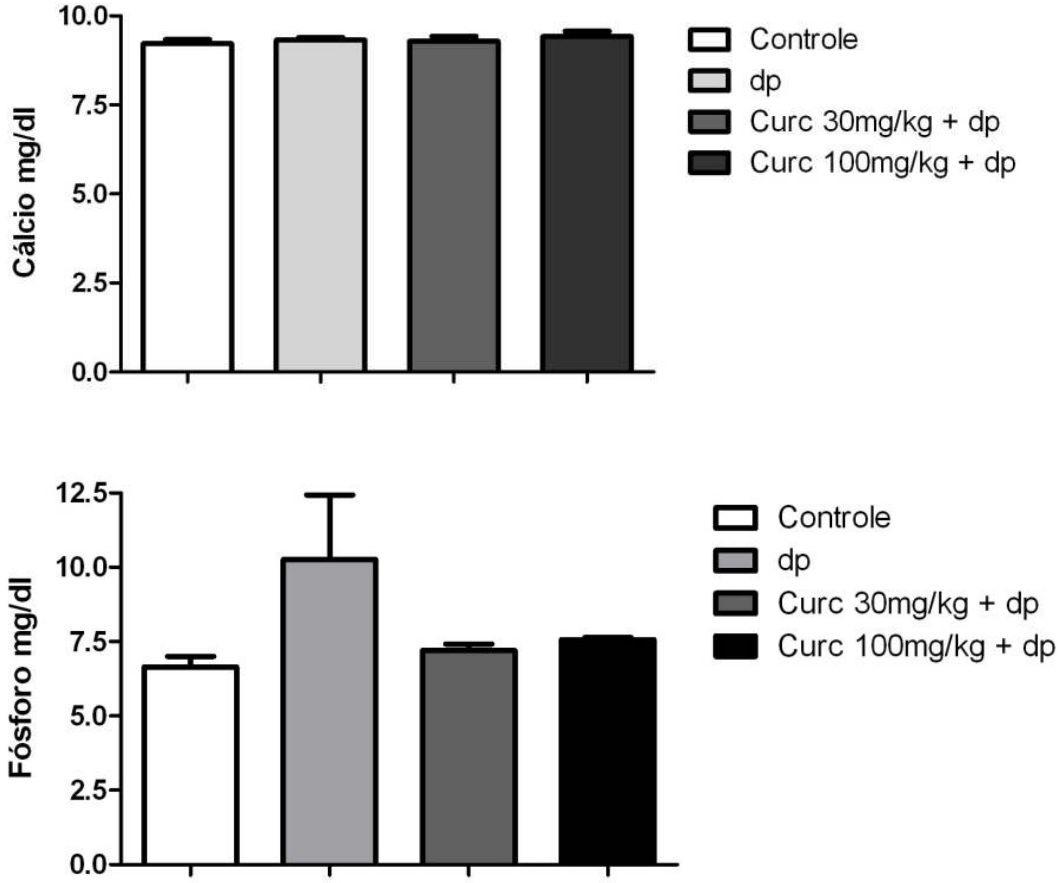
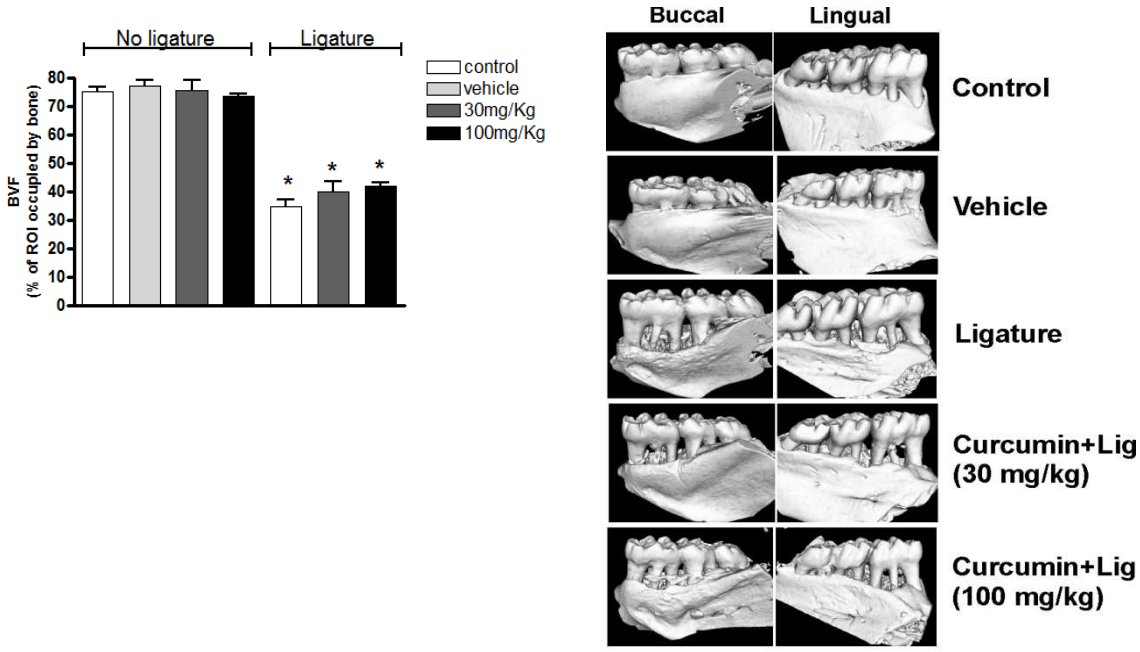


FIGURA 4: Os gráficos mostram os níveis séricos de cálcio e fósforo nos animais segundo os grupos experimentais (controle e doença periodontal), tratados ou não com curcumin. A indução da doença periodontal não causou redução dos níveis de Cálcio e a administração diária do curcumin também não provocou alterações. A discreta elevação dos níveis de Fósforo observada com a indução de doença periodontal foi prevenida pelo tratamento com curcumin. As barras representam medias e as linhas verticais desvios-padrão calculados a partir dos dados obtidos de um mínimo de 6 animais por grupo experimental.

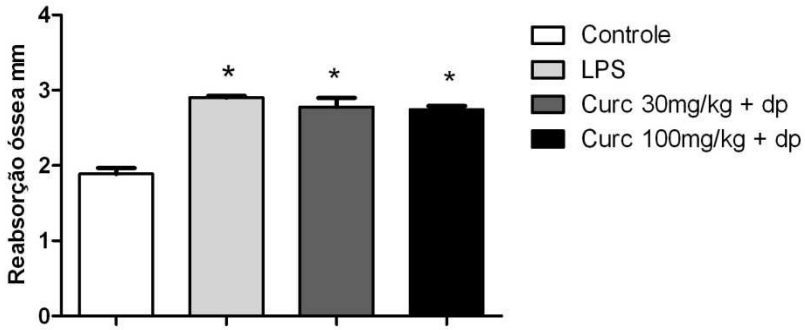
Curcumin não previne a reabsorção óssea associada aos dois modelos de experimentais de doença periodontal

A reabsorção óssea foi avaliada nos molares inferiores através da microtomografia computadorizada (Figura 5, painel A) e nos molares superiores através da técnica de coloração com azul de metileno (Figira 5, painel B). Em ambas as avaliações curcumin não inibiu a reabsorção estimulada pela indução da doença periodontal experimental.

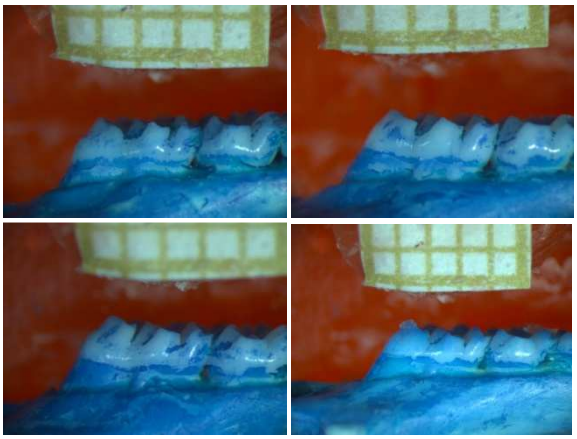
A



B



Controle LPS



Curc 30 mg/kg + LPS **Curc 100 mg/kg + LPS**

FIGURA 5: A administração do curcumin não teve efeito sobre a reabsorção óssea alveolar nos dois modelos de indução da doença periodontal. No painel A o gráfico mostra que a fração de volume ósseo (BVF) foi significantemente diminuída nos dentes com ligadura indicando que as ligaduras induziram reabsorção óssea. O tratamento com curcumin, entretanto, não preveniu a reabsorção. (* indica diferença estatística, $p< 0.05$, em comparação ao controle). No painel B, o gráfico representa os valores da área de reabsorção óssea em milímetros. A injeção de LPS induziu uma reabsorção óssea em relação ao grupo controle que foi estatiscamente significante (* indica diferença estatística, $p< 0.05$, em comparação ao controle). Também neste modelo a administração do curcumin não inibiu a reabsorção experimentalmente induzida. Nos gráficos, as barras indicam médias e as linhas verticais os desvios-padrão calculados a partir dos dados obtidos de um mínimo de 3 animais por grupo experimental.