UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Angélica Letícia Reis Pavanelli

Efeito do tratamento sistêmico com tanshinona na modulação da doença periodontal experimental: estudo em camundongos

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia - Área de Concentração em Periodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista Júlio de Mesquita Filho, como pré-requisito para obtenção do título de Mestre em Odontologia.

Orientador: Prof. Dr. Rafael Scaf de Molon

Coorientador: Prof. Dr. Joni Augusto Cirelli

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Comissão julgadora

Dissertação para obtenção do título de Mestre em Odontologia

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DADOS CURRICULARES

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*Romanos 8,28**

* Bíblia de Jerusalém. Romanos, 8, 28. Nova edição, revista e ampliada. 10ª impressão. São Paulo: Paulus, 2002

Pavanelli ALR. Efeito do tratamento sistêmico com tanshinona na modulação da doença periodontal experimental: estudo em camundongos [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

Resumo

A periodontite é uma doença inflamatória crônica, altamente prevalente, de etiologia multicausal e que se desenvolve como resultado de complexas interações parasita-hospedeiro. Se não tratada, ela resulta na reabsorção do tecido conjuntivo mineralizado e não mineralizado ao redor dos dentes. O processo inflamatório próximo ao esqueleto leva a um fenótipo clínico de perda óssea, observado em diversas patologias, como artrose, artrite reumatoide e doença periodontal (DP). Esse processo é dependente da diferenciação e atividade de osteoclastos, as células responsáveis por reabsorver o tecido ósseo. Desta forma, a inibição da diferenciação ou da atividade dessas células é uma estratégia viável para o controle da reabsorção óssea. A Tanshinona é uma substância natural da *Salvia miltiorrhiza* (Danshen) que emergiu recentemente como fármacos promissores na redução do fenótipo osteoporótico em camundongos. Tais compostos são capazes de inibir especificamente a atividade colagenolítica da catepsina K, enzima secretada pelos osteoclastos que participa da degradação da fase orgânica da matriz óssea, sem interferir na diferenciação de osteoclastos. Porém, os efeitos desses fármacos na inibição da perda óssea causada por inflamação não estão bem elucidados. Sendo assim, o presente estudo apresenta como objetivo avaliar os efeitos desses fármacos como tratamento sistêmico na modulação da DP experimental. Para isto, foram utilizados camundongos C57BL6/J, machos distribuídos em 4 grupos experimentais (n=10), conforme descritos: grupo doença periodontal (DP), grupo tanshinona TIIA (TIIA), grupo tanshinona T06 (T06), e o Grupo Controle (C). Os animais dos grupos DP, TIIA e T06 receberam indução experimental de DP por meio da colocação de ligaduras. As tanshinonas foram administradas diariamente por 10 dias para o grupo TIIA e T06, e o Grupo DP obteve o tratamento com solução veículo (água) por meio de gavagem oral. Após esse período os animais foram eutanasiados. As amostras obtidas foram utilizadas para avaliação do processo imuno-inflamatório presente no tecido gengival, por meio de análise de RT-PCR em tempo real. A avaliação da perda óssea alveolar foi avaliada por microtomografia computadorizada (µCT). O grupo DP apresentou significante perda óssea alveolar, no período de 10 dias, comparado ao grupo controle e os demais grupos TIIA e T06. Houve aumento significante na expressão gênica do mediador inflamatório IL-1b e na catepsina K envolvida no processo de osteoclastogênese para o grupo DP em relação ao grupo controle. Essas expressões se mostraram menores nos grupos TIIA e T06 comparados ao grupo DP. Com base nos achados do presente estudo, sugere-se que as tanshinonas (TIIA e T06) são estratégias promissoras na modulação da DP experimental em camundongos.

Palavras - chave: Catepsina K. Doenças periodontais. Perda do osso alveolar. Osteoclastos.

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Abstract

Periodontitis is a highly prevalent chronic inflammatory disease of multicausal etiology that develops as a result of complex host parasite interactions. If left untreated, it results in the resorption of mineralized and non-mineralized connective tissue of the teeth. The inflammatory process near the skeleton leads to a clinical phenotype of bone loss, observed in several pathologies, such as arthrosis, rheumatoid arthritis and periodontal disease (PD). This process is dependent on the activity of osteoclasts, the cells responsible for the resorption of the bone tissue. This process is dependent on the differentiation and activity of osteoclasts, the cells responsible for resorbing bone tissue. Thus, inhibition of differentiation or activity of these cells is a viable strategy to control bone resorption. Tanshinone is a natural substance from Salvia miltiorrhiza (Danshen) that has appeared as promising drugs in reducing the osteoporotic phenotype in mice. Such compounds are capable of specifically inhibiting the collagenolytic activity of cathepsin K, an enzyme secreted by osteoclasts that participates in the degradation of the organic phase of the bone matrix, without interfering with osteoclast differentiation. However, the effects of these drugs in inhibiting bone loss caused by inflammation are not well understood. Therefore, the present study aims to evaluate the effects of these drugs as a systemic treatment in the modulation of experimental PD. For this, C57BL6/J male mice were distributed into 4 experimental groups (n=10), as described: periodontal disease (PD) group, TIIA tanshinone group (TIIA), T06 tanshinone group (T06), and the control Group. (C). The animals in the PD, TIIA and T06 groups received experimental induction of PD through the placement of ligatures. The tanshinones were administered daily for 10 days for the TIIA and T06 groups, and the DP group was treated with vehicle solution (water) through oral gavage. After this period, the animals were euthanized. The samples obtained were used to evaluate the immuno-inflammatory process present in the gingival tissue by means of real-time RT-PCR analysis. Assessment of alveolar bone loss was evaluated by micro computed tomography (μCT). The PD group showed significant alveolar bone loss within 10 days, compared to the control group and the other TIIA and T06 groups. There was a significant increase in the gene expression of the inflammatory mediator IL-1b and in the cathepsin K involved in the osteoclastogenesis process for the PD group compared to the control group. These expressions were lower in the TIIA and T06 groups compared to the PD group. Based on the findings of the present study, it is suggested that tanshinones (TIIA and T06) are a promising strategy in modulating experimental periodontal disease in mice.

Keywords: Cathepsin K. Periodontal diseases. Alveolar Bone Loss. Osteoclasts.

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal (DP) é uma doença inflamatória crônica, de origem multicausal que se desenvolve a partir de complexas interações parasito-hospedeiro associado a presença de um biofilme disbiótico, e que se caracteriza pela destruição das estruturas de suporte do dente como gengiva, ligamento periodontal, osso alveolar e cemento[1, 2]. O início e a progressão da DP estão relacionados a múltiplos fatores etiológicos e de risco, sendo o biofilme bacteriano o principal fator etiológico e o desequilíbrio entre uma infecção localizada e uma resposta inflamatória exagerada do hospedeiro, desempenha papel importante na determinação dos danos as estruturas de suporte dentário[3]. A periodontite é a principal causa de perda dentária em todo o mundo e um fator de risco significativo para doenças sistêmicas, como diabetes, doenças cardíacas, artrite reumatoide, obesidade, aterosclerose[3, 4].

Diversas moléculas e citocinas pró-inflamatórias desempenham papéis importantes no processo da DP, como por exemplo a Interleucina-1b (IL-1b) e o Fator de Necrose Tumoral-α (TNF-α). A IL-1b pertence à família IL-1 e é produzida principalmente por monócitos, macrófagos e células dendríticas. IL-1b induz a ativação transcricional do gene Factor Nuclear kappa B (NF-kb) para expressar moléculas de adesão e citocinas. Também aumenta a expressão de moléculas de adesão endotelial, promovendo o acúmulo de outras células inflamatórias no endotélio[5]. TNF-α além de inibir e matar células tumorais, pode estimular a resposta inflamatória e aumento da expressão de outras citocinas pró-inflamatórias. Linfócitos estimulados por antígenos como células B e T também são importantes no processo da DP. As funções dos linfócitos T incluem ativar fagócitos, causar apoptose em células infectadas e auxiliar as células B, que além de produzir anticorpos, também são responsáveis por apresentar antígenos às células T[6, 7].

Na doença periodontal, o processo reabsorção óssea pode ser observado com o avanço da doença. Esse processo é constituido de várias etapas que começa com a proliferação de precursores de osteoclastos imaturos, depois a diferenciação e maturação desse tipo de célula e, finalmente, a degradação da matriz orgânica e inorgânica do tecido ósseo[8]. A osteoclastogênese é mediada por diversos eventos incluindo moléculas pró-inflamatórias e citocinas assim como o envolvimento do eixo RANKL/RANK/OPG, os quais realizam funções em lesões patológicas envolvendo inflamação crônica. O RANKL trata-se de uma proteína importante na

osteoclastogênese, pois ele liga-se ao RANK na superfície dos pré-osteoclastos e osteoclastos, motivando a diferenciação dos pré-osteoclastos e a ativação dos osteoclastos maduros, impossibilitando assim a apoptose dos osteoclastos maduros[9, 10]. A OPG, uma proteína solúvel que age como receptor e tem propriedades adversa ao RANKL, acaba impedindo sua ligação ao RANK, e consequentemente a diminuição da osteoclastogênese. RANKL e OPG super regulados tem se mostrado envolvidos em diversas doenças, como osteoporose, artrite reumatoide, DP. Na doença periodontal quando um desequilíbrio entre essas citocinas ocorre, ocasiona uma produção maior de RANKL, resultando em um aumento da reabsorção óssea e consequentemente na perda dos dentes[11].

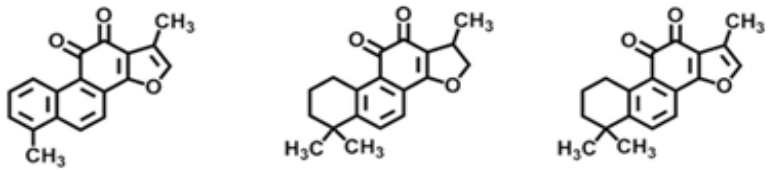
O tratamento da DP inclui mudança de comportamento por parte do paciente como instruções de higiene bucal, cessação do tabagismo, controle de doenças sistêmicas e sequencialmente, o tratamento periodontal não cirúrgico. Este consiste em raspagem e alisamento radicular (RAR) para remoção da placa e cálculo afim de controlar a infecção periodontal e interromper a progressão da doença[12, 13]. No entanto, mesmo após a conclusão do tratamento não cirúrgico, alguns pacientes podem não apresentar um resultado satisfatório, necessitando assim do uso de terapias adjuvantes. Estudos têm investigado o uso de fármacos que modulam a resposta imune, como terapia adjuvante para as doenças periodontais[14, 15].

A catepsina K é uma protease cisteína que está envolvida na regulação da reabsorção óssea e pode ser encontrada em osteoclastos. Elas degradam colágeno tipo I e tipo II, componentes predominantes da matriz extracelular óssea[16, 17]. Estudos têm evidenciado que poder controlar a expressão da catepsina K frente a reabsorção óssea, pode gerar um efeito terapêutico satisfatório para o sistema ósseo durante a progressão da periodontite[18, 19]. Agentes moduladores da resposta imune tem se destacado recentemente devido a sua ação inibitória frente a catepsina K, como o odanacatib, e os inibidores de cistatinas[20-22].

As administrações de compostos naturais como terapia complementar para as DPs têm se mostrado favorável no decorrer dos últimos anos e estudos avaliando os efeitos benéficos do curcumin, fitocistatina, e suplementação com ômega-3 na modulação da resposta imune do hospedeiro, apontaram seus efeitos anti-inflamatório, antirreabsortivo e reparador, o que pode classificar tais compostos naturais como possíveis abordagens terapêuticas adjuvantes para doenças periodontais[23-27], como por exemplo a *Salvia miltiorrhiza*, que é uma planta

originaria da China e suas raízes podem ser utilizadas após serem secas, obtendo-se um pó. Seu uso segundo a tradicional medicina chinesa pode ser direcionado para o coração, fígado, para tratar distúrbios menstruais, reumatismo, osteoporose, entre outras doenças[28]. As Tanshinonas são quinonas diterpenóides lipossolúveis isoladas das raízes secas da *Salvia miltiorrhiza*[29]. As principais tanshinonas isoladas são: 15,16-di-hidrotanshinona (DT), tansinona I (TI), criptotansinona (CT) e tansinona IIA (TIIA)[30, 31]. Estudos têm investigado a ação das tanshinonas[32-34] sendo que um deles relatou que a hidrotanshinona (DT) poderia bloquear seletivamente a atividade da colagenase da Catepsina K sem interferir na atividade da protease e osteoclastogênese[32]. Já a TIIA e CT em um experimento in vitro mostraram que possuem efeito anti-inflamatório devido a sua ação em inibir a ativação da via do fator nuclear kappa B (NF-κB)[33], apontando assim o potencial das tanshinonas como um agente anti-osteoporótico.

Figura 1 - Estrutura molecular das principais Tanshinonas



Fonte: Elaboração própria.

Recentemente, um derivado solúvel da Tanshinona IIA (TIIA), a Tanshinona-IIA sulfonato (T06), foi sintetizado e caracterizado em relação à sua atividade de inibir a reabsorção óssea[34]. Tal composto se mostrou eficaz na redução do fenótipo osteoporótico em camundongos fêmeas ovariectomizadas, sem inibir a diferenciação dos osteoclastos, o que torna esse composto bastante promissor[34]. Em contraste, estudo prévio utilizando a Tanshinona IIA demonstrou o potencial deste composto em inibir osteoclastogênese[35]. Sua eficácia também foi observada em um estudo in vitro utilizando-se macrófagos da medula óssea de camundongos estimulados por lipopolissacarídeo (LPS). A TIIA inibiu a geração de espécies reativas de oxigênio, destacando sua ação anti-inflamatória por meio da regulação metabólica e redox[36]. Há estudos sobre a ação efetiva da TIIA na inibição da

atividade da renina e na redução da expressão de angiotensina II (ANG II), uma enzima que desempenha função na indução do desenvolvimento de osteopenia e osteoporose. Tal redução contribuiu para a melhora na densidade mineral óssea em camundongos diabéticos[37]. Já na consolidação de fraturas, estudo in vitro e in vivo demonstraram que a TIIA possui efeito benéfico, contribuindo para o aumento da deposição de cálcio, atividade de fosfatase alcalina (ALP), colágeno tipo I e aumento da área de calo ósseo. Esses resultados demonstram que a TIIA tem ação positiva também sobre os osteoblastos[38].

O mecanismo de proteção da TIIA na diferenciação de osteoclastos foi avaliado por Cheng et al[39] em camundongos C57BL/6 fêmeas com ovariectomia. Esse estudo identificou que o tratamento com tanshinona IIA inibiu a ativação mediada por RANKL das vias de sinalização de NF-κB, MAPK (Proteínas Quinases Ativadas por Mitogênio) e Akt (Proteína quinase B) durante a osteoclastogênese[39].

Embora alguns estudos in vitro e in vivo tenham indicado as propriedades anti-inflamatórias e anti-osteolíticas da administração sistêmica das tanshinonas, especialmente para o tratamento de doenças ósseas metabólicas como a osteoporose[28, 37, 40], nenhum estudo, até o momento, têm demonstrado os efeitos da administração sistêmica das tanshinonas sobre a reabsorção óssea inflamatória, como no caso das doenças periodontais. Portanto, considerando as características sítio-especifica e a natureza inflamatória da DP, e a capacidade antirreabsortiva e anti-inflamatória da Tanshinona, este estudo foi delimitado com o objetivo de avaliarmos o efeito do tratamento sistêmico das tanshinonas TIIA e T06 na modulação da doença periodontal experimental em camundongos.

2 PROPOSIÇÃO

2.1 Objetivo geral

O objetivo geral desse estudo foi avaliar o potencial das tanshinonas TIIA e T06 em inibir a reabsorção óssea e o processo inflamatório em modelo de periodontite induzido por ligadura em camundongos.

2.2 Objetivos específicos

Determinar o efeito das tanshinonas TIIA e T06 na progressão da perda óssea experimental induzida pela colocação de ligadura por meio de análise microtomográfica. Foi avaliado a densidade mineral óssea (BMD), a microarquitetura trabecular (Tb.Th, Tb.N, Tb.Sp) e o volume ósseo (BV/TV) nas maxilas.

Avaliar a influência das tanshinonas sobre a expressão de marcadores de reabsorção óssea (catepsina K, IL-1ß, TNF-a e RANKL) em nível de mRNA, através de RT-qPCR.

3 REVISÃO DE LITERATURA

Diversos estudos tem demonstrado os benefícios das tanshinonas perante as doenças inflamatórias[41, 42, 43] como a osteoartrite ou artrose que é uma doença que se caracteriza pelo desgaste da cartilagem articular e por alterações ósseas e que está associada também ao envelhecimento que envolve inflamação crônica[44], degradação da matriz na cartilagem e formação anormal de aglomerados de condrócitos[45]. Essa doença limita os movimentos do paciente e atividades simples como camilhar, movimentar os braços, afetando a qualidade de vida. Os tratamentos atuais para a osteoartrite visam o controle da dor e em casos mais severos pode ocorrer a substituição da articulação afetada por meio de cirurgias[46] .

Nesse contexto, Wang et al. avaliaram a ação da tanshinona I na osteoartrite, utilizando células de condrócitos CHON-001. As células CHON-001 foram tratadas com IL-1β (10 ng / mL) durante 72 horas para induzir o modelo de osteartrite, depois foram pré-tratadas com 20 μM de Tansinona I por 24 horas e em seguida estimuladas com IL-1β (10 ng / mL) por mais 72 horas. Análises foram realizadas por meio de ensaios de CCK-8, imunofluorescência, citometria de fluxo e Western blotting. Os resultados mostraram que a Tansinona I inibiu a degradação da cartilagem articular in vitro. O estudo mostrou que a expressão de NF-κB estava aumentada em células CHON-001 estimuladas por IL-1β, e foram revertidos por meio do tratamento com Tansinona I, apontando assim, que a Tanshinona I pode aliviar a progressão da osteoartrite por meio da supressão do nível de NF-κB[42].

Já Zhang et al. avaliaram o efeito protetor da tansinona IIA contra a desdiferenciação de condrócitos. O estudo foi realizado com condrócitos coletados das articulações dos joelhos de ratos, utilizando o método de digestão com colagenase. Os condrócitos foram cultivados em meio basal com várias concentrações de TIIA, 100, 200 e 400 μg / mL. Foram realizadas análises histológicas e de RT-qPCR e os resultados mostraram que a TIIA inibiu a regressão de condrócitos e ocasionou proliferação e a viabilidade dos condrócitos nas concentrações para todas as concentrações. O estudo sugere que o efeito da TIIA pode ser mediado por vários mecanismos regulatórios como SOX6, um fator de transcrição importante para o desenvolvimento da cartilagem[37]. Jia et al. também avaliaram a tanshinona TIIA na degradação da cartilagem articular em um modelo de osteoartrite de rato e demonstraram que a tansinona IIA preveniu a degradação

da cartilagem articular por meio da inibição da apoptose de condrócitos e secreção de citocinas inflamatórias e também aumentou os níveis de expressão de TGF-β (Fator de crescimento transformador beta) e de proteínas morfogenéticas ósseas[47].

Medicamentos como prednisona, dexametasona são glicocorticóides muito utilizados devido aos seus efeitos anti-inflamatórios[48]. Estudo relatam que a ingestão de glicocorticóides em altas doses e por um longo período, acaba agindo como um dos principais contribuintes para apoptose de osteócitos[49, 50]. Li et al. avaliaram a hipótese de que TIIA antagoniza a apoptose induzida por glicocorticóides, por meio da inibição da produção de ROS em células MC3T3-E1 (pré-osteoblastos de murinos) e investigaram também, qual mecanismo é responsável por esse efeito. O estudo buscou fornecer uma nova estratégia para a prevenção contra a osteoporose induzida por glicocorticóides. Assim sendo, utilizaram células MC3T3-E1 e a apoptose de osteoblastos foi induzida por dexametasona. Após a indução, realizaram o tratamento com 1 μM de TIIa por 24 h. Análises por meio de ensaios de MTT e Tunel, Western blot, ROS e análise de apoptose por citometria de fluxo foram realizadas nas amostras. Os resultados mostraram que a TIIA inibiu a apoptose celular induzida por glicocorticóide por meio da inativação de Nox4 (enzima NADPH oxidase) que é uma enzima expressa abundantemente em osteoblastos, mostrando assim os benefícios das tanshinonas como possível farmaco para doenças que causam de reabesorção óssea[51].

A osteoclastogênese é o processo pelo qual as células da linhagem hematopoiética se diferenciam em osteoclastos maduros. Um desequilíbrio na ação dos osteoclastos favorece uma maior reabsorção óssea, o que pode levar a osteoporose, uma doença óssea sistêmica que causa perda e deterioração óssea, levando à fragilidade óssea e risco de fraturas[52, 53]. No processo da reabsorção óssea podemos destacar três moléculas principais; o Receptor Ativador do Fator Nuclear Kappa (RANK), Ligante do Receptor Ativador do Fator Nuclear Kappa(RANKL) e Osteoprotegerina(OPG)[54]. RANK trata-se de um receptor expresso por pré-osteoclastos e osteoclastos. Já o RANKL é uma proteína produzida por osteoblastos e células estromais da medula, ele se liga ao RANK na superfície dos pré-osteoclastos e osteoclastos, induzindo assim a diferenciação das células progenitoras ósseas e estimulando a função dos osteoclastos maduros. OPG é um receptor regulador da sinalização de RANKL e atua bloqueando a ligação com seu receptor RANK, interferindo assim no processo de

diferenciação dos osteoclastos. Compreender as vias de sinalização e mecanismo da osteoclastogênese é importante para que se possa avaliar possíveis alvos terapêuticos para tratamento de doenças que acometem o tecido ósseo[55, 56].

O Denosumab é um farmaco que atua de forma semelhante ao OPG, que é um receptor para RANKL. Denosumab se liga ao RANKL e o impede de se ligar ao seu receptor RANK, de modo que a via de sinalização RANK não é ativada, prejudicando a diferenciação, função e ocasionando apoptose de precursores de osteoclastos, resultando na inibição da reabsorção óssea[57]. Os Bisfosfonatos diferentemente do Denosumab, atuam nos osteoclastos e não nos seus precursores. Eles são internalizados em osteoclastos e inibem a FPP sintase, uma enzima chave na via de sinalização do mevalonato. Isso resulta em prenilação proteica intracelular prejudicada, afetando a função dos osteoclastos e levando à apoptose, dessa forma, a reabsorção óssea é inibida[58]. No entanto, a aplicação clínica dos Bisfosfonatos é limitada devido o risco de desenvolvimento de osteonecrose dos maxilares após sua administração[59]. Outro farmaco que apresentou notáveis resultados perante a inibição da reabsorção óssea foi o Odanacatib, inibindo a Catepsina K (CatK), uma protease degradadora de colágeno ósseo em osteoclastos. Dessa forma o Odanacatib manteve a formação óssea sem alterações, porém seus ensaios clínicos foram encerrados em razão ao aumento dos efeitos colaterais cardiovasculares associados, como derrames[60-62].

Panwar e colaboradores em um estudo buscando um inibidor para doenças de reabsorção óssea criaram um novo conceito de inibição para a CatK. Nesse novo conceito denominado ectosterico que difere de alostérico, ocorre a inibição seletiva da catepsina K e não da célula toda (osteoclasto), pois observaram que produzir um composto muito seletivo como o caso Odanacatib, não foi vantajoso uma vez que, isso afetou as demais funções da CatK[34]. Assim sendo Panwar at al. realizaram um estudo com a Tanshinona IIA sulfonato (T06) em camundongos fêmeas ovariectomizadas, para analisarem os efeitos da T06 como inibidora da CatK. Os animais receberam por meio de gavagem a T06 na dosagem de 40 mg / kg durante 3 meses. Após esse período, os animais foram sacrificados e o tecido do útero, fêmur esquerdo e a vértebra lombar (L5) foram coletados para análises. O fêmur direito e o tecido do útero foram analisados por histologia, o sangue também foi coletado por punção cardíaca e usado para determinar a concentração plasmática de CTx-1 por meio de ELISA, a parte óssea também foi analisada por Micro-Ct. Os

resultados mostraram um aumento de volume ósseo e maior número trabecular nas regiões analisadas, assim como aumento no número de osteoblastos por perímetro ósseo e da concentração plasmática de pró-colágeno-1. Isso devido a ação que a T06 exerceu em bloquear a atividade da colagenase da catepsina K, exercendo a atividade antirreabsortiva[34].

Cui et al. também avaliaram o tratamento com tanshinona em ratas fêmeas com osteoporose induzida por ovariectomia e os dados obtidos nesse estudo sugerem que os efeitos protetores da tanshinona diminuíram a superfície de osteoclastos nas ratas ovariectomizadas devido a supressão da reabsorção óssea aumentada pela depleção de estrogênio. Os animais receberam uma dosagem de 200 mg/kg de tansinona total, administrada por via oral por um período de 10 semanas. Após o periodo foram sacrificados e a quarta vértebra lombar (L4) e metáfises proximais da tíbia foram coletadas para análises por meio de histomorfométria óssea e histologia. Os resultados mostraram um aumento de volume ósseo / volume de tecido e redução de osteocalcina / superfície óssea e da porcentagem de superfície de osteoclastos na quarta vértebra lombar e nas metáfises proximais da tíbia[63]. Outro estudo que avaliou os efeitos da TIIA em ratas Sprague Dawley com osteoporose induzida por ovariectomia, apontou que a possível regulação positiva de PHGDH pederia ser suficiente para atrasar o envelhecimento celular e suprimir a osteoporose primária[64].

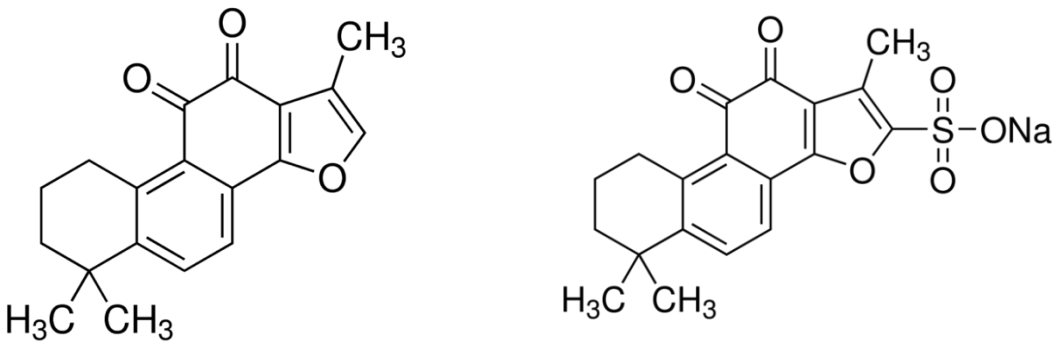
O uso das tanshinonas tem se mostrado uma alternativa interessante para inibição da perda óssea. Todos os estudo ralizados com as tanshinonas podem ajudar a modular a resposta inflamatória do hospedeiro e melhorar a progressão da doença experimental, podendo ser um farmáco futuro para o tratamento de doenças que causam perda óssea como a doença periodontal.

4. MATERIAL E MÉTODOS

4.1 Tanshinonas

A tanshinona TIIA (T4952) e a tanshinona T06 (sódio sulfonato) (SML2517) foram obtidas diretamente da empresa Sigma-Aldrich. A tanshinona-sulfonato (T06) é um derivado solúvel da tanshinona IIA diferindo-se apenas pela adição dos canais de potássio na molécula, conforme demonstrado nas imagens abaixo.

Figura 2 – Estrutura molecular da Tanshinona TIIA e T06.



Tanshinona TIIA Tanshinona IIA sulfonato (T06)

Fonte: Elaboração própria.

4.2 Desenho experimental

Para este estudo foram utilizados 40 camundongos C57BL6/J obtidos do biotério da Faculdade de Medicina de Botucatu (FM – UNESP) com 8 semanas de idade e com peso médio de 18-25g. Ao longo do período experimental, os camundongos foram mantidos nas instalações para animais do biotério da faculdade de Odontologia da UNESP de Araraquara com temperatura controlada (23° C ± 2° C), e humidade controladas, e com um ciclo de claro/escuro de 12 horas. Os animais foram alojados em gaiolas de plástico, alimentados com uma dieta de laboratório (Labina / Purina) e com água ad libitum.

Os animais foram randomicamente distribuídos em 4 grupos experimentais, conforme descrito abaixo:

- *Grupo C (controle)* – Os animais não receberam nenhuma intervenção cirúrgica e nenhuma intervenção terapêutica;

- *Grupo DP* – Os animais receberam indução da DP por meio da colocação de ligaduras e foram submetidos ao tratamento com solução veículo (água);

- *Grupo TIIa* – Os animais receberam indução de DP por meio da colocação de ligaduras e foram submetidos ao tratamento diário com TIIa (40mg/kg);

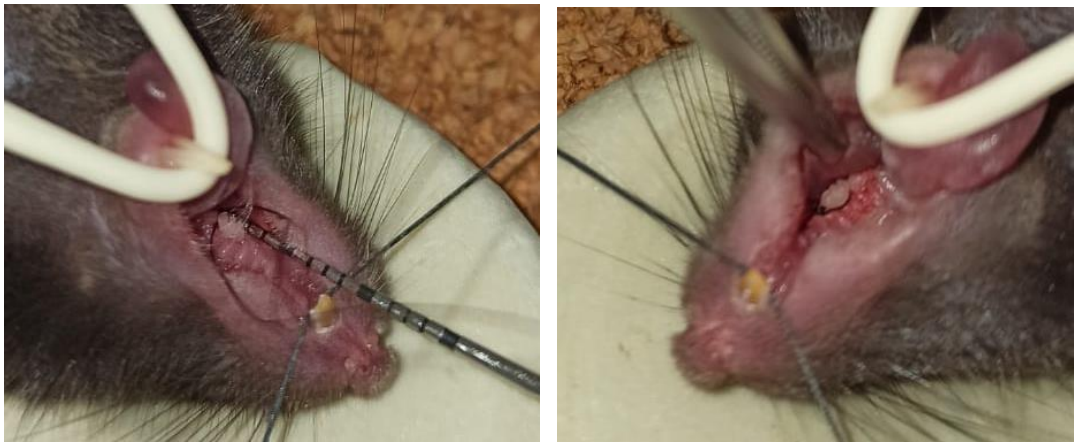
- *Grupo T06* – Os animais receberam indução de DP por meio da colocação de ligaduras e foram submetidos ao tratamento diário com T06 (40mg/kg).

As drogas foram administradas por gavagem 1x ao dia, durante todo o curso do experimento. A via de administração e dosagem foi escolhida baseada em estudos anteriores com TIIA (35).

4.3 Indução da doença periodontal

A indução da DP pela colocação de ligaduras foi realizada como descrito previamente[65,66]. Após a anestesia geral com Quetamina (80 mg/kg) e Xilasina (10 mg/kg) administrada por injeção intramuscular, os animais foram posicionados em decúbito dorsal sobre o aparato cirúrgico e, com auxílio de um suporte, a boca permaneceu aberta ao longo da manipulação. Com a boca aberta e língua imobilizada, uma sonda periodontal foi inserida entre o primeiro e segundo molar superior a fim de proporcionar espaço para a colocação do fio. Assim, com o uso de uma pinça adaptada, o fio de nylon (Ethicon 6.0) foi inserido entre os molares e, após seu posicionamento correto, foi amarrado ao redor da cervical do dente (Figura 3). O ressecamento da língua e dos olhos do animal, devido ao tempo que permaneceu parado com os olhos e boca abertos, foi controlado com irrigação com solução salina (NaCl 0,9%). As ligaduras foram instaladas no início do experimento. Todas as ligaduras permaneceram na sua posição e não houve necessidade de recolocação.

Figura 3 - Procedimento para indução da DP por meio da colocação de ligaduras



Fonte: Arquivo pessoal da autora.

4.4 Administração das tanhinonas TIIA e T06 (sódio sulfonato)

Para a diluição da Tanshinona T06 (5mg/ml) foi utilizada água destilada (2,3ml), aquecida a 45°C e posteriormente agitada com auxílio do vórtex por 2 minutos. A Tanshinona TIIA (5mg/ml) foi diluída utilizando polissorbato 20 (Tween 20) (15 % volume/volume), etanol (2 % v/v) e água q.s.p (quantidade suficiente) em 2ml. A mistura foi homogeneizada em vórtex e mantida em banho ultrassônico por 10 minutos até o momento do seu uso.

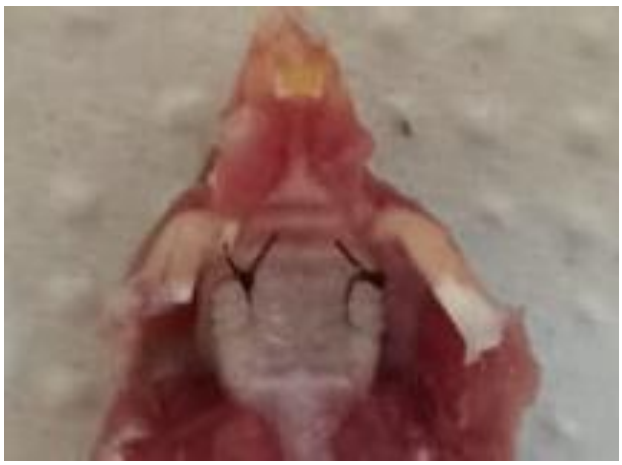
Para os procedimentos de administração do medicamento por gavagem, foi utilizada uma cânula de metal (cânulas de gavagem para camundongo em inox com diâmetro de 1mm com esfera de 1,7 mm, raio de 40 mm e comprimento de 31 mm) e 200µL de cada uma das doses das tanshinonas ou da água destilada foram administradas diariamente, 1 vez ao dia, durante 10 dias, iniciando-se no mesmo dia da instalação das ligaduras.

4.5 Sacrifício e coleta das amostras

Após o período experimental de 10 dias, os animais de cada um dos grupos experimentais foram sacrificados por deslocamento cervical (Figura 4). As maxilas foram cuidadosamente removidas, e subdivididas em duas hemimaxilas, no qual a hemi-maxila do lado esquerdo teve o tecido gengival ao redor dos primeiros e segundos molares removidos para extração de mRNA (qPCR em tempo real). Para esta finalidade, os tecidos coletados 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. As hemi-maxilas do lado direito foram fixadas em

parafolmaldeído 4% por 48h, e transferidas para etanol 70% para avaliação da perda óssea por microtomografia computadorizada.

Figura 4 - Momento após o sacrifício dos animais e coleta da maxila



Fonte: Arquivo pessoal da autora.

4.6 Análise microtomográfica

Antes do escaneamento, as amostras da maxila foram lavadas em água destilada e armazenadas em solução salina a 0,9% durante a noite para reidratação das peças. As amostras ósseas foram envoltas em papel húmido para evitar artefatos como consequência da desidratação durante o processo de digitalização. Amostras ósseas da maxila foram escaneadas utilizando-se um sistema de microtomografia computadorizada (μCT Skyscan 1176; Skyscan, Kontich, Belgium). Os parâmetros de escaneamento foram escolhidos com base em trabalhos publicados anteriormente pelo nosso grupo de pesquisa[65, 66]. Brevemente, os parâmetros utilizados foram: gerador de raios-X operado a 50 kVp, feixe de corrente em 500 μA, filtração de alumínio de 0,5 mm de espessura em uma resolução de imagem de 12,45 μm; passo de rotação de 0,5°. As amostras foram colocadas no microtomógrafo em um cilindro de espuma sob a cama de carbono. Para reconstrução das amostras (NRecon 1.6.1.5; Bruker), os seguintes parâmetros foram utilizados: defect-pixel masking: 10%; beam-hardening: 20%, smoothing: 1, e calculado individualmente os valores de compensação de desalinhamento.

Após as reconstruções, para a realização das análises lineares, dados volumétricos foram importados no software Data Viewer (Skyscan, Kontich, Belgium) para geração de imagens reconstruídas multiplanares. Na maxila, a distância da

junção cemento-esmalte até a crista óssea alveolar foi mensurada na superfície mesial e distal do primeiro molar e mesial do segundo molar utilizando-se cortes sagitais.

Análises volumétricas foram realizadas após a aquisição das mensurações lineares. Para isso, volume ósseo (BV), tecido ósseo (TV) e fração de volume ósseo (BV/TV) foram analisados utilizando-se o software CTAn (CT Analyzer 1.12.0.0 - Skyscan, Kontich, Belgium) em cortes axiais (1024 x 1024). Todos as imagens foram salvas no Dataviewer (Dataviewer 1.4.3 – Skyscan, Kontich, Belgium). Para isso, uma região de interesse (ROI) foi delineada a partir dos ápices das raízes até a crista alveolar, e a partir da raiz mesial do primeiro molar até a raiz distal do segundo molar. Em detalhe, o ROI consistiu em 60 cortes na maxila da porção apical do dente até a junção cemento-esmalte. O limite inferior foi definido quando apareceram os ápices das raízes mesial do primeiro molar e distal do terceiro molar. O tecido ósseo foi manualmente desenhado em intervalos regulares com um método baseado em fatia a cada 10 planos utilizando-se uma ferramenta interpolada. Subsequentemente, as raízes foram desenhadas manualmente nos 60 cortes selecionados e foram excluídos do osso. Assim, a área óssea inteira sem as raízes e ligamento periodontal foi incluída no ROI. Tons de cinza para quantificação dos parâmetros foram determinados usando o algoritmo de limiarização. Usando processamento personalizado, manchas brancas (menores que 30 voxels) foram removidas.

Mensurações da arquitetura óssea na maxila também foi realizada utilizando o software CTAn para avaliação do número de trabéculas (Tb.N), separação de trabéculas (Tb.Sp) e espessura de trabéculas (Tb.Th). A densidade mineral óssea (BMD) também foi avaliada. Para isso, a calibração do software CTAnalyzer foi realizada pela determinação dos valores de hidroxiapatita de cálcio (CaOH) dos dois phantoms contendo 0,25 e 0,75 g de CaOH/cm^3. Os phantoms foram escaneados de acordo com os parâmetros utilizados para o escaneamento e reconstrução das maxilas, conforme previamente descrito[24].

4.7 Reação de polimerase em cadeia (RT-qPCR)

O RNA total foi extraído de amostras de tecido gengival usando o kit RNeasy 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. 142,5 ng de RNA total foram utilizados para a síntese de cDNA utilizando random hexamers como primers e seguindo as instruções do fabricante (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 3,5 µL que inclui Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) para cada gene: (catepsina K, IL-1ß, TNF-a e RANKL) e a expressão de GAPDH foi utilizada para normalização dos resultados, que foram analisados pelo método delta (delta Ct).

Os genes alvos, Assay ID #, Acession # e amplicon de cada TaqMan Gene Expression Assay estão descritos na Tabela 1.

Tabela 1 – Informações sobre os conjuntos de primers TaqMan pré-otimizados para PCR em Tempo Real (TaqMan Gene Expression Assays, Applied Biosystems)

Gene alvo	Assay ID	Acession #	Amplicon (bp)
GAPDH	Mm 99999915 gl	NM_001289726.1	107
Ctsk	Mm00484039_m1	NM_007802.4	73
IL-1b	Mm01336189_m1	NM_008361.3	63
TNF – α	Mm00443259_g1	NM_013693.3	81
RANKL	Mm01205928_ml	NM_011613.3	75

Fonte: Elaboração própria.

4.8 Análise estatística

Para o estudo in vivo, o cálculo do tamanho da amostra foi calculado utilizando-se o software G*Power 3.1 e foi baseado em estudo previamente publicado[111]. A Reabsorção inflamatória do osso alveolar foi estabelecida como sendo o desfecho primário do estudo. O tamanho do efeito foi calculado considerando um erro alpha de 0.05 (α=0,05 – erro do tipo II) e poder de teste de 80% (1-β=0,80 – erro tipo I) para detectar uma diferença de aproximadamente 10% de perda óssea alveolar entre o grupo teste e controle. Assim, o tamanho do efeito foi determinado como sendo de 2.91. O número mínimo de 7 animais por grupo foi

considerado necessário. Considerando que poderia haver alguma morte inesperada, 10 animais por grupo foram incluídos.

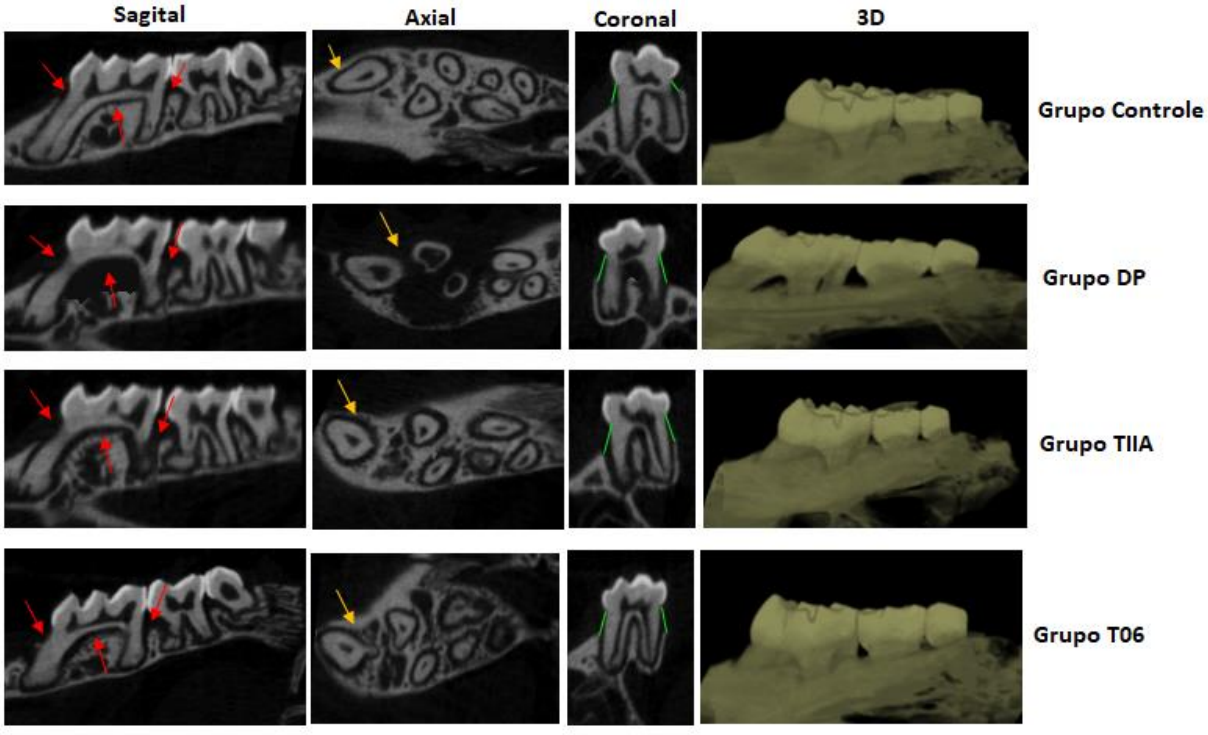
Os dados obtidos nas diferentes análises foram analisados utilizando-se o software GraphPad Prism 7.0 (GraphPad Software Ind., San Diego, CA, EUA). O objetivo da análise foi comparar os resultados de cada desfecho de acordo com os tratamentos. A normalidade dos dados foi verificada utilizando-se o teste de Shapiro-Wilk. De acordo com a distribuição dos dados, foi utilizado ANOVA com teste post-hoc de Tukey para comparações pareadas, ou o teste de Kruskal-Wallis seguido pelo teste de Dunn para dados não paramétricos. O nível de significância foi estabelecido em 5% ($p < 0,05$) em todas as análises.

5 RESULTADOS

5.1 Análise de µCT

Para analisar a quantidade de perda óssea nos grupos com doença periodontal, foi medida a distância da junção cemento-esmalte (JCE) à crista alveolar (CA) na superfície mesial e distal do primeiro molar e mesial do segundo molar. Quando se observa um aumento da JCE-CA pode-se dizer que a um aumento da perda óssea. A análise linear demonstrou que houve aumento na distância JCE-CA significativa para todos os grupos doentes, sendo a maior distância observada no grupo DP quando comparados com os demais grupos (Figura 5 A). A fração de volume ósseo (BV/TV) foi significativamente diminuída no grupo DP em comparação com o grupo Controle, TIIA e T06 (Figura 5 B). A densidade óssea alveolar (BMD) não apresentou diferenças significativas entre o grupo Controle, TIIA e T06 quando comparados com o grupo DP o qual obteve uma maior perda estatisticamente. (Figura 5 C).

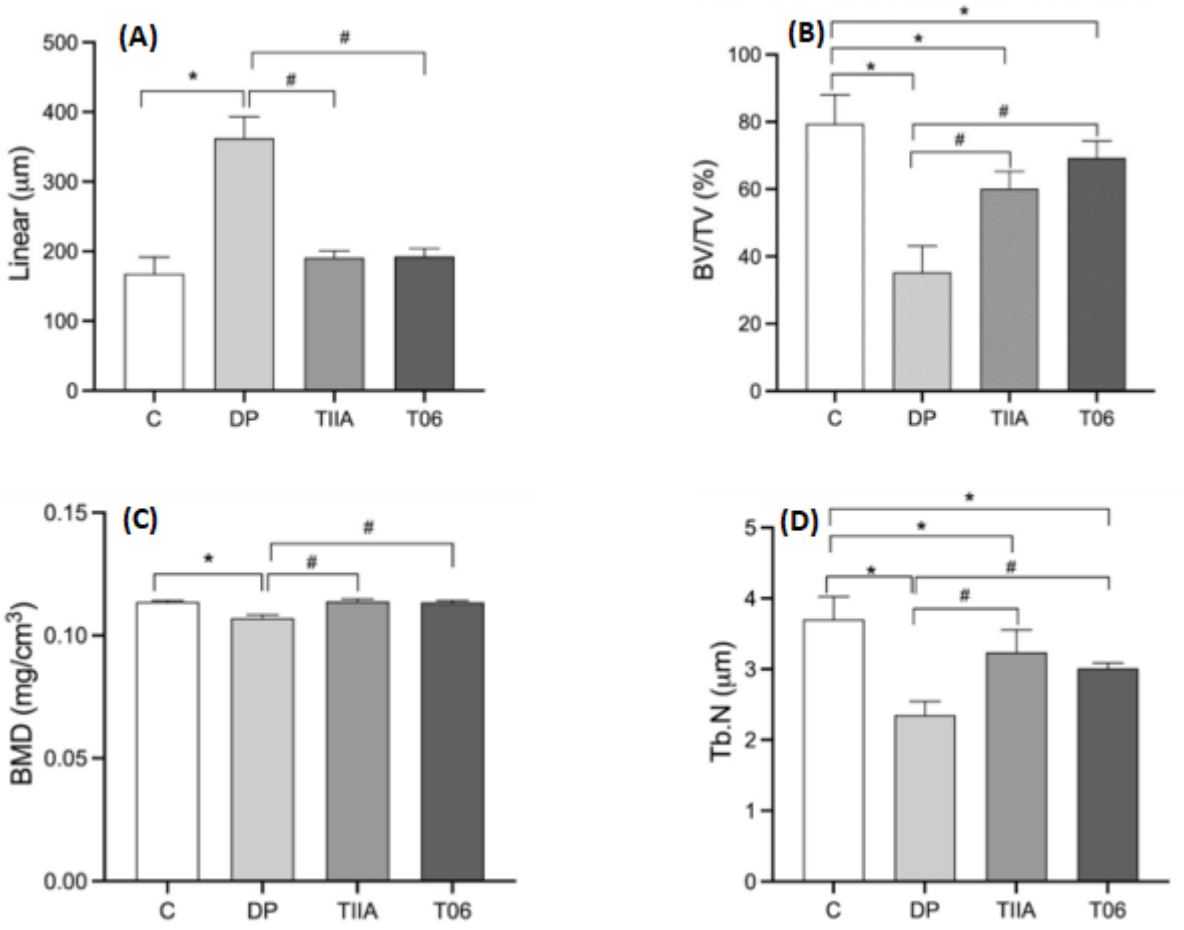
Figura 5 - Imagens representativas de cada grupo experimental obtidas nos planos sagital, axial, coronal, e plano 3D

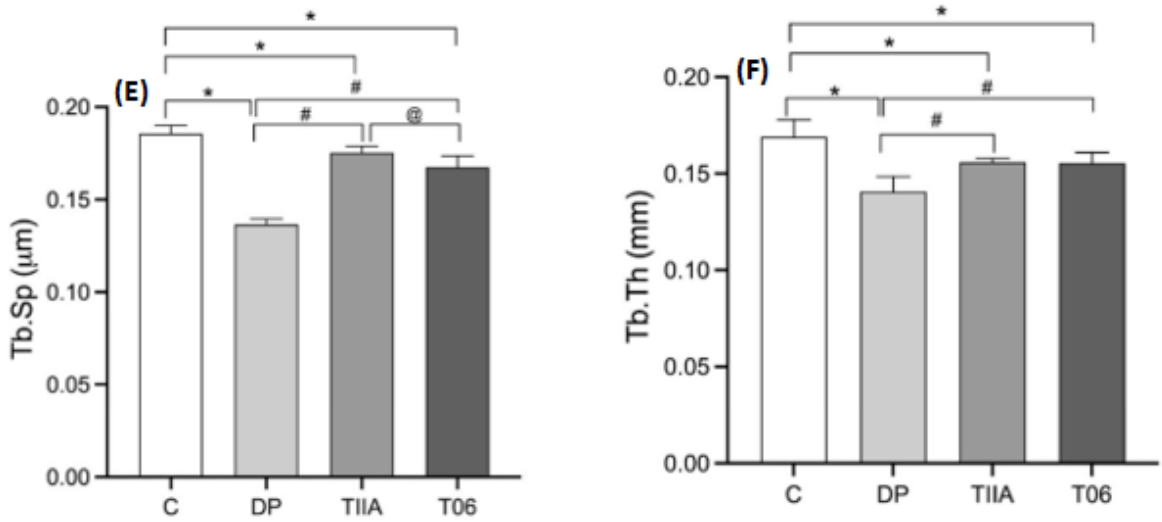


Fonte: Elaboração própria.

As mensurações da arquitetura óssea na maxila realizada para avaliação do número de trabéculas (Tb.N), separação de trabéculas (Tb.Sp) e espessura de trabéculas (Tb.Th) apontaram que o Tb.N se mostrou estatisticamente menor no grupo DP quando comparado com grupo Controle. Pode-se notar uma diferença relativamente menor também entre o grupo Controle e o grupo TIIA e T06 (Figura 5 D). Para Tb.Sp o grupo DP se mostrou menor quando comparado aos demais grupos. Já o grupo das tanshinonas TIIA e T06 também apresentaram valores menores para separação de trabéculas comparados ao grupo Controle e houve uma diferença estatisticamente entre a TIIA e a T06 (Figura 5 E). Diferente de Tb.Th, onde o grupo TIIA e T06 não apresentaram diferenças estatísticas entre si, apenas quando comparadas com o grupo Controle e grupo DP o qual mostrou maior perda para espessura de trabéculas (Figura 5 F).

Figura 6 - Análise gráfica da perda óssea alveolar linear (A), da fração de volume ósseo (BV/TV) (B), densidade óssea alveolar (BMD) (C), do número de trabéculas (Tb.N) (D), da separação de trabéculas (Tb.Sp) (E) e da espessura de trabéculas (Tb.Th) (F).





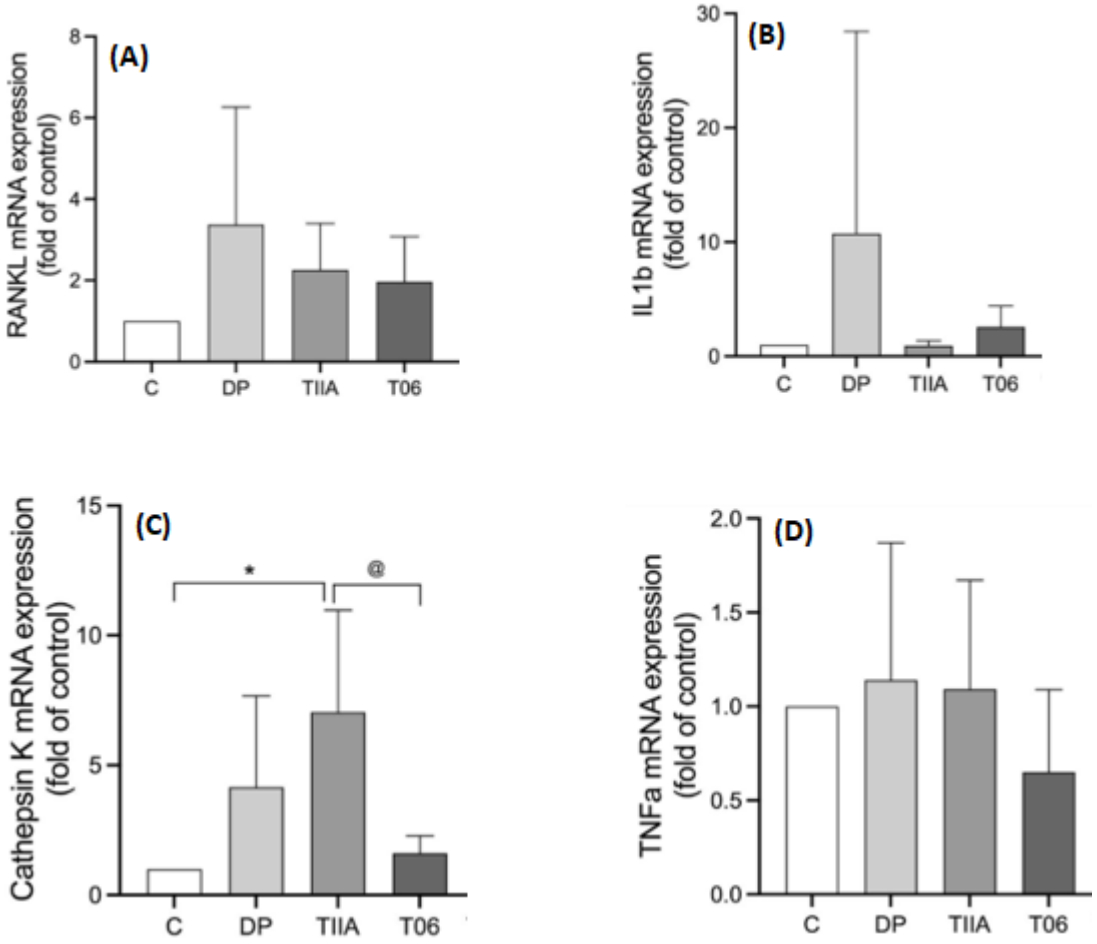
*Diferença estatistica do grupo controle (p <0.05) #Diferença estatistica do grupo DP (p <0.05) @Diferença estatística do grupo T06 (p <0.05).

Fonte: Elaboração própria.

5.2 RT-qPCR

A expressão dos genes RANKL e IL 1b foi maior no grupo DP em relação aos demais grupos, e pudemos observar uma diferença concisa entre os grupos TIIA e T06 para RANKL (Figura 7A). Por outro lado, o grupo tanshinona (TIIA e T06) apresentou os menores valores de expressão de IL1b em relação ao grupo DP, e o grupo TIIA foi o mais próximo do grupo controle (Figura 7B). No entanto, o grupo T06 apresentou menor expressão gênica de catepsina K e TNFa em relação aos demais grupos (Figura 7 C e D).

Figura 7 - Análise gráfica RT-qPCR das expressões de RANKL (A), IL1b (B), Catepsina K (C), TNFa (D).



*Diferença estatistica do grupo controle (p <0.05) @Diferença estatística do grupo TIIA (p <0.05).

Fonte: Elaboração própria

6 DISCUSSÃO

As tanshinonas têm sido estudadas atualmente devido ao seu potencial de inibição da reabsorção óssea em doenças metabólicas crônicas como a osteoartrite, osteoporose e artrite reumatoide[29, 35, 37]. Trabalhos anteriores mostraram que o mecanismo de ação das tanshinonas age na resposta imuno-inflamatória bem como através da inibição da enzima Catepsina K, os quais inibem especificamente a sua atividade colagenolítica nos osteoclastos[32, 34]. Porém, há uma escassez de estudos que avaliam a ação das tanshinonas no processo de reabsorção óssea inflamatória, como nas DPs. Com base nesse fato, nosso trabalho teve como objetivo avaliar in vivo o efeito do tratamento sistêmico com tanshinona na modulação da doença periodontal experimental.

De maneira geral, os resultados do presente estudo demonstraram que tanto a administração sistêmica da tanshinona IIA, bem como da tanshinona sulfonato T06 resultou em uma redução estatisticamente significante da perda óssea alveolar em camundongos submetidos a DP experimental. Além disso, a expressão genica de IL-1b, uma citocina pró-inflamatória relacionada diretamente com a perda óssea alveolar, foi significativamente diminuída após o tratamento sistêmico com tanshinona IIA e T06. Houve também uma redução da expressão gênica de RANKL nos grupos tratados, mas que não demonstraram significância estatística. Esses resultados sugerem que as tanshinonas possuem um efeito positivo na modulação da resposta inflamatória e óssea na DP experimental, nas condições estudadas.

A análise da perda óssea por meio de microtomografia computadorizada demonstrou que os grupos administrados com tanshinona tiveram uma redução em torno de 50% na perda óssea alveolar quando comparado com o grupo DP. Além disso, houve um aumento na densidade mineral óssea nos grupos tratados, os quais obtiveram valores semelhantes aos do grupo controle (sem doença). Embora estudos anteriores não tenham sido realizados objetivando avaliar os efeitos do tratamento sistêmico da tanshinona durante a progressão da perda óssea alveolar, um estudo pré-clínico em camundongos com osteoporose induzida demonstrou que esse tratamento sistêmico resulta na diminuição da perda de massa óssea e da densidade mineral óssea[34]. Embora a osteoporose e a DP tenham etiologia completamente distintas, ambas resultam na perda de massa óssea por meio de um

desequilíbrio entre formação e reabsorção óssea, havendo um desequilíbrio favorecendo o processo de reabsorção. Nesse contexto, o uso das tanshinonas para modular a perda óssea pode ser considerada como uma alternativa viável para o tratamento de doenças ósseas crônicas.

A inibição da perda óssea alveolar encontrada neste estudo pode estar relacionada com a diminuição da expressão genica de IL-1b e RANKL. Durante a progressão da periodontite, esses mediadores pró-inflamatórios são produzidos em grande escala e são amplamente disseminados no organismo, os quais influenciam o metabolismo e a resposta imuno-inflamatória[67-69]. Estudos anteriores relataram que a tanshinona IIA age na diferenciação osteogênica de células-tronco do ligamento periodontal por meio de indução Runx2 dependente de ERK1/2[36], já a tanshinona T06 pode bloquear a atividade colagenolítica da Catepsina K, exercendo a atividade antirreabsortiva[34], o que pode ser observado em nossa análise de RT-qPCR, onde a T06 se mostrou mais eficaz para diminuir a expressão genica de Catepsna K e TNFa quando comparada aos demais grupos. Outro fator que pode evidenciar a ação inibitória das tanshinonas na reabsorção óssea é a sua regulação perante a ação dos osteoclastos, isso ocorre por meio de um ajuste da razão do fator de diferenciação da osteoprotegerina (OPG) e osteoclastos[70].

Neste estudo foi administrado uma dosagem de 40 mg/kg de TIIA e T06 diariamente durante todo o período experimental, de acordo com estudo previamente descrito na literatura[34]. É importante destacar que análise histológica e imunohistoquimica estão sendo conduzidas no momento para a avaliação do processo imuno-inflamatório e para a marcação de osteoclastos e catepsina K para elucidar os fatores que podem contribuir para diminuição da perda óssea alveolar no modelo de periodontite experimental.

Portanto, embora os resultados micro-tomográficos tenham sido favoráveis a inibição da perda óssea inflamatória, é importante mencionar que este trabalho ainda está em andamento e que novas metodologias para avaliação do processo inflamatório estão sendo desenvolvidas para avaliar através de quais mecanismos esses compostos bloqueiam a perda óssea alveolar. Outro fator que deve ser levado em consideração é que este estudo apresenta algumas limitações às inferências clínicas. O modelo animal utilizado não mimetiza propriamente o que acontece na DP em humanos. Além disso, fatores de interferência importantes como a influência dos fatores de risco sistêmicos e a progressão aguda do modelo de perda óssea

alveolar, precisam ser levados em consideração em estudos futuros.

Por fim, estudos comparando diferentes dosagens para as tanshinonas devem ser realizados, bem como em diferentes modelos de perda óssea inflamatória (como nos modelos osteolíticos em calvaria), a fim de nos ajudar a entender o comportamento farmacológico das tanshinonas.

Outro fator que poderá ser investigado é a possibilidade de utilizar diferentes vias de administração das tanshinonas, com potencial para uso local com dispositivos de liberação lenta e controlada, para atuar de maneira mais eficaz no tratamento de uma doença de natureza sitio-especifica. Essas questões devem ser abordadas em estudos futuros.

7 CONCLUSÃO

De acordo com os dados apresentados nesse estudo, sugere-se que as tanshinonas TIIA e T06 são eficazes na modulação da doença periodontal experimental in vivo, nas condições estudadas.

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ANEXO A – COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara

FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 19/2020

Araraquara, 24 de fevereiro de 2020.

Senhor Pesquisador:

A Comissão de Ética no Uso de Animal - CEUA desta Faculdade procedeu a análise do Relatório Parcial do projeto de pesquisa de sua responsabilidade intitulado ***"TANSHINONAS: INIBIDORES DE CATEPSINA K COMO POTENCIAIS FÁRMACOS PARA O TRATAMENTO DA DOENÇA PERIODONTAL"*** (Proc. CEUA nº 19/2020), e considerou-o APROVADO, bem como sua solicitação de alteração na metodologia e no modelo animal.

Lembramos que o Relatório Final deste projeto deverá ser entregue em **ABRIL/2022.**

Atenciosamente.

Profa. Dra. CARINA APARECIDA FABRICIO DE ANDRADE
Coordenadora da CEUA

Ao
Prof. Dr. JONI AUGUSTO CIRELLI
DD. Pesquisador Responsável
Departamento de Diagnóstico e Cirurgia

APÊNDICE A – PUBLICAÇÃO*

Pharmacological therapies for the management of inflammatory bone resorption inperiodontal disease. A review of pre-clinical studies

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Abstract

Periodontitis, a highly prevalent multicausal chronic inflammatory and destructive disease, develops as a result of complex host-parasite interactions. Dysbiotic bacterial biofilm in contact with the gingival tissues initiates a cascade of inflammatory events, mediated and modulated by the host's immune response, which is characterized by increased expression of several inflammatory mediators such as cytokines and chemokines in the connective tissue. If periodontal disease (PD) is left untreated, it results in the destruction of the supporting tissues around the teeth, including periodontal ligament, cementum, and alveolar bone, which leads to a wide range of disabilities and poor quality of life, thus imposing significant burdens. This process depends on the differentiation and activity of osteoclasts, the cells responsible for reabsorbing the bone tissue. Therefore, the inhibition of differentiation or activity of these cells is a promising strategy for controlling bone resorption. Several pharmacological drugs that target osteoclasts and inflammatory cells with immunomodulatory and anti-inflammatory effects, such as bisphosphonates, anti-RANK-L antibody, strontium ranelate, cathepsins inhibitors, curcumin, flavonoids, specialized pro-resolving mediators, and probiotics were already described to manage inflammatory bone resorption during experimental PD progression in pre-clinical studies. Meantime, a growing number of studies have described the beneficial effects of herbal products in inhibiting bone resorption in experimental PD. Therefore, this review summarizes the role of several pharmacological drugs used for PD prevention and treatment and highlights the targeted action of all those drugs with antiresorptive properties. In addition, our review provides a timely and critical appraisal for the scientific rationale use of the antiresorptive and immunomodulatory medications in pre-clinical studies, which will help to understand the basis for its clinical application.

Keywords: Alveolar bone; animal model; antiresorptive agents; bone; periodontal disease; osteoclast.

Introduction

Periodontal disease (PD), a chronic inflammatory condition of the supporting tissues around the teeth, is characterized by the loss of supporting structures of the tooth, such as gingiva, periodontal ligament, alveolar bone and cementum [1-3]. This condition leads to an irreversible loss of the dental structures and might result in teeth loss if left untreated [1, 4]. The etiology of PD is multifactorial in which the presence of a dysbiotic biofilm in intimate contact with the gingival margin initiates the inflammatory immune response [3, 5]. Indeed, PD is the sixth-most prevalent disease globally [6] and is considered the most important cause of teeth loss in the adult population [7].

PD is modulated and mediated by the immune host system, which plays an important role in disease severity and progression. During the initiation and progression of PD, environmental conditions (smoking), systemic comorbidities (diabetes mellitus and rheumatoid arthritis), and genetic polymorphisms (IL-1ß) are important factors involved in [8-10]. In the initiation phase of PD, there is an activation of the inflammatory response, which is characterized by increased vascular permeability of the junctional epithelium, increased gingival crevicular fluid, and an influx of inflammatory cells (leukocytes), especially the polymorphonuclear neutrophils (PMN) that tends to diminish the insult caused by the dysbiotic biofilm [4]. All of these events are protective, and in most patients, the immune system is capable of controlling the disease progression. However, innate and adaptive responses in susceptible patients lead to the aggravation of periodontal tissue destruction. The activation of leucocytes and T cells in the connective tissue lead to the production of multiple inflammatory mediators, degrading enzymes such as matrix metalloproteinases (MMP), and the increased expression of the nuclear factor-kappa B ligand (RANKL), which is the primary activation factor for osteoclasts [11], leading to periodontal inflammation and finally cause the loss of bone supporting tissue (Figure 1) [12-14].

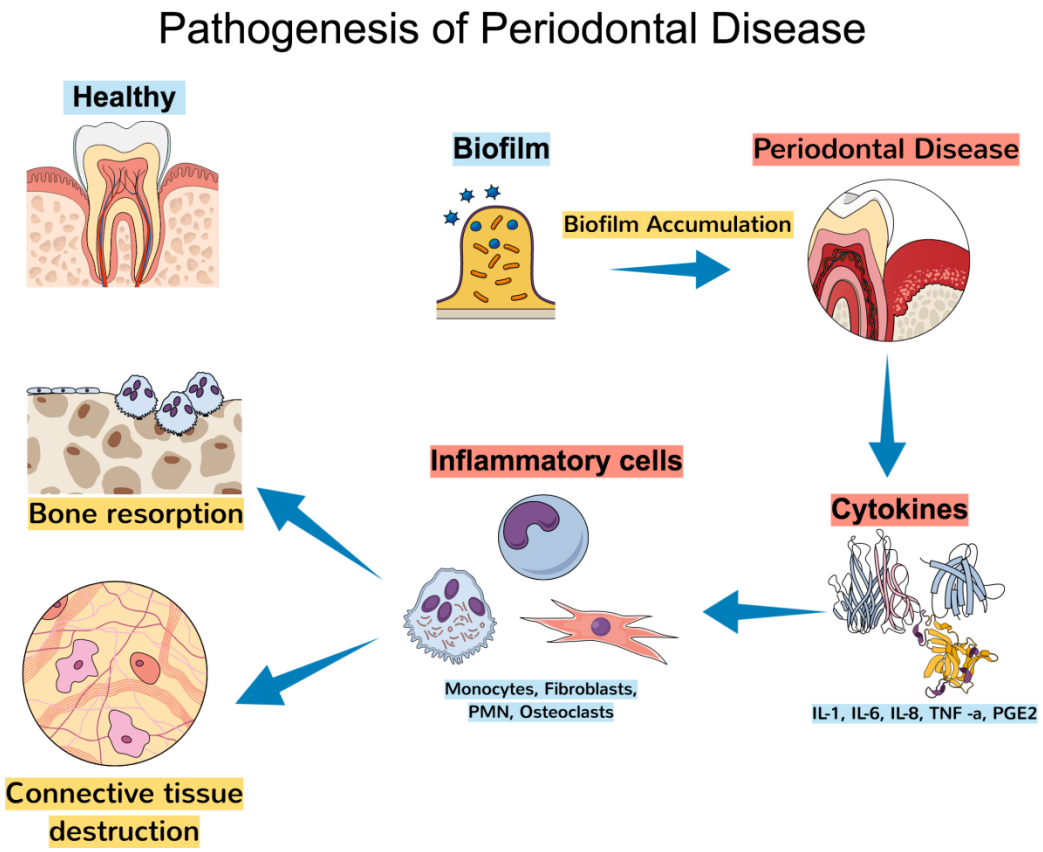

Fig. 1 - The pathogenesis of PD. The bacteria that compose the dental biofilm trigger the process of local inflammation generated by the increase of cytokines such as IL-1, IL-6, IL-8, TNF-a, PGE2 and inflammatory cells. Such inflammatory environment leads to the activation of osteoclasts, the cells responsible to resorb the bone tissue. Consequently, the signs and symptoms of PD occurs.

The primary treatment of PD is through scaling and root planning (SRP) to remove the attached biofilm from the root surface. However, removing bacterial biofilm does not imply a return to homeostasis and regeneration of lost tissues [15, 16], and SRP targeting only microorganisms does not accomplish favorable results in all patients [17]. Adjunctive treatments such as systemic local antibiotics, non-steroidal anti-inflammatory drugs, and low doses of doxycycline have been used as host modulating agents in order to control the progression of PD [18-21]. Despite the clinical benefits of those approaches, their effects are limited in the context of inflammation-induced alveolar bone loss [22]. The major challenge for successfully treating PD is the difficulty in finding a target that can inhibit tissue

inflammation and consequently alveolar bone destruction [23]. Therefore, the adjunct use of complementary therapies that aim to modulate the destructive events of the immune response has been proposed as a potential therapeutic strategy for PD treatment targeting inflammatory mediators and bone-resorbing osteoclasts.

In recent decades, the use of pharmacological drugs and natural compounds (herbal medicine) aiming to suppress bone destruction during experimental PD in animal models has been extensively reported [24-33]. Interestingly, several studies have shown that inhibition of bone loss can be targeted intervened by innumerous pharmacological drugs, such as alendronate [34-37], OPG-Fc [25], resolvin [38-41], strontium ranelate [26, 42], Curcumin [16, 30, 43-46], cathepsins inhibitors [28, 47], and others. Therefore, in this review, we comprehensively summarize the roles of several therapeutic drugs during the progression of PD, and provide the main findings of each included study leading to the prevention of experimental PD.

In this review, the pharmacological products discussed below are examined through many experiments for their anti-osteoclastic activity. The in vivo studies included in this review are based on well-established experimental models of PD, such as ligature-induced bone loss [48-52], lipopolysaccharide (LPS) injections [53-55], and oral inoculation of periodontopathogenic bacteria into the animal mouth [49, 53-55]. Primary methods used to evaluate the inhibition of bone loss were assessed by microcomputed tomography (micro-CT) and histopathological analyses. Oral gavage, palatal injections and intraperitoneal injections represent the major intervention method in in vivo testing. Humanized mouse models, subcutaneous bacterial injections or other animal models were not investigated in this review. We have described the objective, study design, main findings and conclusions of all the included studies, according to Table 1-9.

Cathepsin K inhibitors

Cathepsin K (CtsK) is a member of the papain superfamily (C1 protein family) of cysteine protease that plays an important role in the innate immune response and osteoclast-mediated bone resorption [23, 56]. It was previously identified as an osteoclast selective protease CtsK [57] abundantly expressed in human osteoclasts, osteoblasts, periodontal ligament cells, osteocytes, and fibroblasts. In the bone tissue, CtsK can cleave the triple helix and the telopeptides from the type I collagen fibers that constitute 90% of the bone organic matrix

[58]. In addition, this protease can also activate MMP-9 [59] and degrade type II collagen [60], osteonectin and osteopontin, thus inhibiting the activity of osteoclasts [58]. It is important to mention that CtsK inhibitors are able to prevent bone resorption without affecting osteoblastic activity. Therefore, the crosstalk between osteoblast and osteoclast is maintained, which is beneficial during bone remodeling [61]. A summary of main study outcomes is described down below (Table 1).

Table 1. **CATHEPSIN K INHIBITORS**

Studies	Study Design	Main outcomes
Yue et al. (2020) [63]	**Animals:** Eighty male DBA / J1 mice (8 weeks old) **Disease model:** Collagen-induced arthritis (CIA) model. Periodontal disease (PD) model with *P. gingivalis infection.* **Treatment:** Injections of Adeno-associated virus (AAV) transfection in periodontal tissue and knee joint. AAVs 2,5 × 10 - 10 g / ml. Given once every 3 days for 65 days.	Inhibition of articular tissue damage and alveolar bone loss, decreased number of macrophages and expression of inflammatory cytokines in the synovia, due to inhibition of CtsK.
Pan et al. (2019) [66]	**Animals:** Twenty 6 to 7 weeks old DBA/1 male wild-type mice. **Disease model:** CIA mouse model and PD model with *P. gingivalis* infection. **Treatment:** Ctsk-specific inhibitor BML-244 (25.242 mg/kg per week) or dimethyl sulphoxide (DMSO; vehicle).	Reduced expression of inflammatory cytokines and infiltration by dendritic cells and T cells. Bone loss in PD and RA abrogated. Inhibition of CtsK decreased Toll-like receptor (TLR) 4 and TLR9 expression in vivo.
Hao et al. (2015) [23]	**Animals:** Seventy-five 8 week old female wild-type BALB/cJ mice. **Disease model:** Bacteria-induced PD model; 100 µL (5×10^9 CFU/mL of *P. gingivalis*; 5×10^8 CFU/mL of *T. denticola* e *T. forsitia*) topical application eight consecutive times. **Treatment:** Orally with 3.606 or 0.7212 mg/kg per week (five times lower dose) of ODN in DMSO for 56 days.	Decreased number of osteoclasts, T cells and macrophages, and toll-like receptors; Decreased bone loss and improved immune response during the progression of PD.
Chen, W et al. (2016) [24]	**Animals:** Twenty-one Wild-type female BALB/cJ mice. **Disease model:** PD induced by oral inoculation with *P. gingivalis*. **Treatment:** Gingival injections in the upper molar region of AAV-sh-Ctsk or AAV-sh-luc-YFP (3 ul) daily for seven consecutive days.	Less bone loss and inflammation in the gingival tissue due to CtsK inhibition.
Da Ponte Leguizamón et al. (2021) [72].	**Animals:** Twenty-four 8 week old C57BL/6J male mice. **Disease model:** Ligature-induced periodontal disease. **Treatment:** CsinCPI-2 (0.8 µg/g in PBS) for 15 days.	Controlled the inflammatory process, inhibited osteoclastogenesis and alveolar bone loss.

Previous studies have described selective CtsK inhibitors that effectively reduce osteoclast resorption both in vitro and in vivo [62-64]. Furthermore, CtsK has been shown to be an efficient therapeutic strategy in pre-clinical studies, including inflammatory, metabolic and

autoimmune diseases, such as high fat acid-induced obese mice [65], experimental periodontitis [23, 24, 66] and collagen-induced arthritis (CIA) [66]. However, although CtsK inhibitor has potent inhibitory effects on osteoclast-mediate bone resorption, it has also been associated with some adverse side effects and undesired drug-drug interactions [61, 66, 67].

Researches have long held that inflammation and bone breakdown are the two major pathological features of periodontitis and rheumatoid arthritis (RA); consequently, prevention or reduction of these damaging events should be a main therapeutic objective. In this regard, Yue et al. [66] recently investigated the effect of CtsK inhibition on the course of a combined animal model of CIA and experimental PD through oral infection with *P. gingivalis*, a known periodontopathogenic bacteria. The results of this study have demonstrated that inhibition of CtsK by transfection of small interfering RNA (siRNA) resulted in diminished destruction of articular tissue and alveolar bone, and decreased macrophage numbers and inflammatory cytokine expression in the synovium, suggesting that CtsK inhibition might be implicated as a potential therapeutic strategy in future research of experimental PD and RA [66].

A previous study also demonstrated that inhibition of CtsK effectively suppresses autoimmune inflammation of the joints as well as osteoclastic bone resorption in autoimmune arthritis [68]. Pan et al. [69] have used an experimental periodontitis model through oral bacterial inoculation combined with CIA in DBA/1J mice. One week before establishing the combined diseases, animals were treated with CtsK inhibitor BML-244. The data demonstrated that the alveolar bone resorption and paw swelling were more severe when these two comorbidities were present simultaneously. Furthermore, inhibition of CtsK reduced inflammatory cytokine production and infiltration by dendritic cells and T cells. Consequently, bone loss in PD and RA was abrogated as measured by bone erosion in periodontal lesions and cartilage destruction in knee joints. Inhibition of CtsK also decreased the expression of Toll-like receptor (TLR) 4 and TLR9 in vivo [69].

As previously stated, CtsK also has functions in dendritic cells through the TLR9, which plays a pivotal role in innate immunity recognition of microbial products and in the

activation of immune host defense [23, 68]. In this context, Hao et al. [23] evaluated whether inhibition of CtsK would benefit both the immune system and bone system during the progression of bacteria-induced periodontitis in a mouse model. A small molecular inhibitor, odanacatib, was orally administered into the mouse mouth one week prior to experimental PD establishment. This study demonstrated that oral application of odanacatib decreased the number of osteoclasts, T cells and macrophages, and toll-like receptors, thus preventing bone loss and exacerbated immune response during the progression of PD [23].

Another study evaluated the inhibition of CtsK through adeno-associated virus (AAV) expressing CtsK small hairpin to silence CtsK [24]. Experimental PD was induced by oral gavage with *P. gingivalis*. AAV-sh-CtsK was administered locally into the palatal gingival tissue. The data demonstrated that inhibiting CtsK drastically protected the mice from *P. gingivalis*-induced bone loss (>80%) and significantly reduced inflammation in the gingival tissue. The authors suggested that inhibition of CtsK can target both inflammation and bone resorption and efficiently protect against periodontal bone destruction.

Indeed, the use of CtsK inhibitors for the treatment of osteolytic diseases remains promising. However, in contrast to current anti-resorptive agents, which target the osteoclast cells, CtsK inhibitors can cause effects in other tissues, as the enzyme is not only present in bone cells but also engages in several other metabolic processes and regulatory pathways. The challenge, then, is to develop more specific inhibitors, which act on the osteolytic activity of the CtsK, without affecting the activity of other enzyme catalytic sites, decreasing the chance of side effects [61, 70].

CtsK activity is regulated by endogenous cysteine proteinase inhibitors, such as cystatin C, which has a high binding affinity to cysteine proteinases [71]. These proteins are capable of inhibiting osteoclastogenesis and bone resorption in vitro and in an ex vivo model [72, 73]. Recently, our group demonstrated that natural inhibitors of cysteine peptidase derived from Citrus sinensis, named phytocystatin CsinCPI-2 was effectively in decrease the gene expression levels of cathepsin K, cathepsin B, IL-1β, and TNF-α. In addition, CsinCPI-2 significantly inhibited in vivo the activity of TNF-α in the blood of rats, previously stimulated by *E. coli* lipopolysaccharide (LPS). These data suggested that CsinCPI-2 has a potential anti-inflammatory effect during bacteria infection in rats [74]. Moreover, we have just showed that the phytocystatin CsinCPI-2 was able to control the inflammatory process

and inhibit osteoclastogenesis and loss of alveolar bone in a mouse model of ligature-induced experimental PD [75].

Bisphosphonates

Bisphosphonates, particularly nitrogen-containing ones, such as zolendronate and alendronate, are antiresorptive agents commonly used to treat bone metabolic diseases such as osteoporosis and bone neoplasia, Paget disease and multiple myeloma. Bisphosphonates inhibit functioning osteoclasts by impairing differentiation, disrupting the cytoskeleton, decreasing intracellular transport, and inducing apoptosis, and does so through the inhibition of farnesyl diphosphate synthase in the cholesterol biosynthesis pathway, which prevents prenylation of small guanosine triphosphatase signaling proteins [76-78]. Some of the described studies is described in table 2.

Table 2. **BISPHOSPHONATES**

Studies	Study Design	Main outcomes
Brunsvold et al. (1992) [84]	**Animals:** 27 adult cynomolgus monkeys with intact dentitions. **Disease model:** PD induced by ligature placed around the lower premolars and molars, plus oral inoculation of *P. gingivalis*. **Treatment:** Alendronate (0.05 mg/kg) for 16 weeks	Decreased the progression of PD as measured by changes in bone density.
Moreira et al. (2014) [35]	**Animals:** Thirty-six 3 months-old Wistar rats. **Disease model:** Ligature-induced PD around the upper right second molar. **Treatment:** Daily injections of 2.5 mg/kg body weight alendronate for 7 days before and 7, 14 and 21 days after PD induction.	Reduced the activity of osteoclasts and the resorption of the alveolar bone crest. After 21 days of treatment, some animals developed signs of ONJ due to reduced osteoclast activity.
Almeida et al. (2015) [34]	**Animals:** Ninety 3-month-old Wistar rats. **Disease model:** Ligature-induced PD around the lower left first molar. **Treatment:** Scaling and root planning and/or administration of Alendronate (irrigation with 1 ml of 10-5 M) for 7, 15 and 30 days.	The combination of the two treatments showed less local inflammation and enhanced tissue repair.

Despite its beneficial effects in inhibiting bone resorption in osteolytic diseases, the use of bisphosphonates, especially intravenous administration of high doses of zolendronate, are associated with adverse side effects. The most significant effect associated with

bisphosphonate administration is the osteonecrosis of the jaw (ONJ), a condition defined as an area of exposed bone in the maxillofacial region that does not heal after 8 weeks in patients receiving antiresorptive therapies [79, 80]. Furthermore, atypical fractures are also related to the long-term use of bisphosphonate due to its high maintenance of the drug into the bone tissue. On the other hand, the oral administration of alendronate to treat osteoporosis has shown to have a 0% to 0.4% chance of inducing ONJ [81]. Consequently, several studies have investigated the beneficial effects of alendronate administration to manage experimental periodontitis in rats [34, 36, 82, 83] and PD in clinical trials [84-86].

One of the first studies that have used alendronate as adjunctive therapy to manage experimental PD was conducted by Brunsvold et al. in 1992 [87]. In this study, the authors have induced experimental PD in monkeys by placing a ligature around the mandibular premolars and molars followed by oral inoculation of *P. gingivalis* one week after alendronate administration. Alendronate was administered intravenously for 16 weeks, and clinical and radiographical analyses were performed. The authors demonstrated that 0.05 mg/kg alendronate treatment reduced the progression of PD, suggesting its use to treat PD. Similarly, Moreira et al. [35] have shown that 2.5 mg/kg alendronate administration in rats with experimental PD reduced the activity of osteoclasts and significantly decreased the resorption of the alveolar bone crest. However, after 21 days of treatment, some animals developed signs of ONJ due to the reduced activity of osteoclast. The authors pointed out that using alendronate to treat experimental PD in rats might increase the risk of ONJ development.

The use of alendronate as adjunctive to scaling and root planning (SRP) in rats with induced PD was evaluated by de Almeida et al. [34]. Rats with ligature induced-PD received SRP after ligature removal associated with topical application of alendronate. The animals assigned to receive SRP plus alendronate showed less local inflammation and better tissue repair, associated with higher expression of osteoprotegerin (OPG) immunolabeling, suggesting that the treatment employed might be effective in the treatment of PD in rats. A recent systematic review investigated the potential use of bisphosphonate as an adjuvant to SRP in 13 clinical trials [88]. The results of this systematic literature review demonstrated

that locally or systemically administered alendronate reduced probing pocket depth and resulted in a gain of clinical attachment level and improved radiographic assessment. Indeed, bisphosphonate as an adjuvant to SRP may result in clinical benefits in patients with PD. However, the risk to ONJ development after bisphosphonate administration limits their clinical use.

OPG-Fc and RANKL inhibitors

The discovery of the RANK, RANK ligand (RANKL) and OPG axis has revealed its pivotal role in regulating bone metabolism and created a new field for the study of bone-related diseases [11]. Binding of RANKL to RANK results in the differentiation and maturation of osteoclast precursor cells to activated osteoclasts. Therefore, blocking the interaction between RANK and RANKL is accountable for inhibiting osteoclast differentiation, and it is considered an interesting alternative to inhibit bone loss in osteolytic lesions. Acting as a soluble decoy receptor for RANKL, OPG binds to RANKL and inhibits osteoclast development preventing it from binding to RANK. OPG has been evaluated in pre-clinical studies of experimental PD as a therapeutic compound for counteracting bone loss (Figure 2).

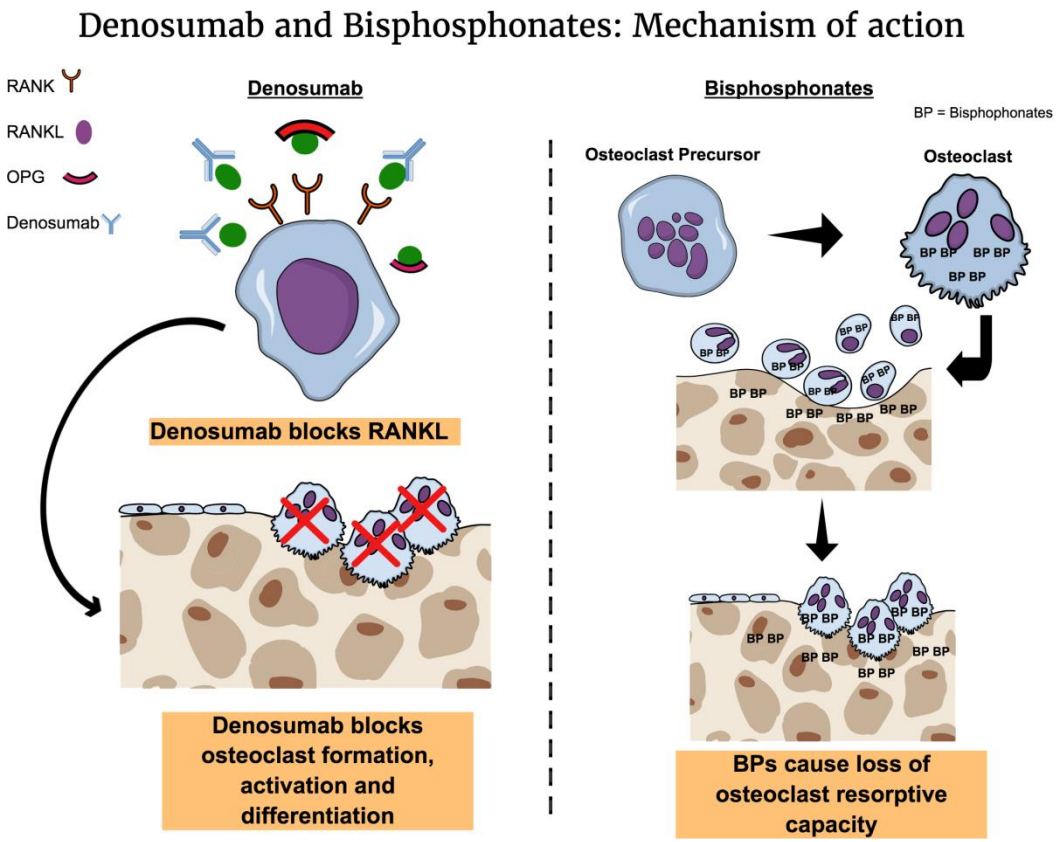

Fig. 2 - Denosumab acts similarly to OPG, which is RANKL's natural decoy receptor; Denosumab binds to RANKL, preventing the binding of RANKL to its receptor, RANK, on the surface of osteoclasts and also on osteoclast precursors. Thus the RANK signaling pathway is not activated, resulting in impaired osteoclast precursor differentiation and function and possibly osteoclast apoptosis. All these effects lead to inhibition of bone resorption. Bisphosphonates act on osteoclasts, but not on their precursors. Bisphosphonates are internalized into osteoclasts possibly by endocytosis. Subsequently, Bisphosphonates inhibit FPP synthase, a key enzyme in the mevalonate signaling pathway. This leads to impaired intracellular protein prenylation impairing osteoclast function and apoptosis. Thus, bone resorption is inhibited.

The pioneering study that has used OPG to treat experimental PD was performed by Teng et al. in 2000 [89]. Using an oral inoculation infection model with *A. actinomycetemcomitans* in mice, the authors demonstrated that in vivo inhibition of RANKL function with OPG treatment reduces alveolar bone loss and decreases the number of osteoclasts after microbial challenge. These data imply that OPG treatment may thus have therapeutic value to prevent alveolar bone and/or tooth loss in human periodontitis. These findings parallel observation made by Mahamed et al. [90] that showed reversal of alveolar bone loss in diabetic mice treated with the RANKL antagonist OPG. Using an acute model of ligature-induced bone loss, Jin et al. [25] demonstrated protective effects of OPG-Fc during experimental PD with significant preservation of alveolar bone. Therefore, OPG revealed robust preventive effects on alveolar bone resorption in experimental PD, thus showing a promising therapeutic potential of OPG for PD treatment.

Moreover, an anti-RANKL monoclonal antibody denominated denosumab has been developed and used to treat bone metabolic diseases such as osteoporosis and metastatic bone cancers and other osteolytic bone conditions such as periodontitis and arthritis. Denosumab binds directly to the RANKL to prevent its interaction with RANK on osteoclasts. This binding inhibits osteoclast formation, differentiation, and function [78], and thus inhibiting bone resorption. Denosumab does not bind to mouse RANKL; therefore, studies have used an anti-mouse monoclonal RANKL to investigate its potential effects on mice. In this context, Kuritani et al. [91] investigated the effects of systemic administration of anti-RANKL during the progression of ligature-induced bone loss in mice. The study

findings showed that anti-RANKL antibody strongly suppressed alveolar bone loss associated with periodontitis in a ligature mouse model. However, similar to bisphosphonates, the potential risk of development of medication-related osteonecrosis of the jaw [92-95], the use of denosumab or RANKL inhibitors as an adjunctive treatment for PD is not indicated. Table 3 depict the main study outcomes with RANK-L inhibitors.

Table 3. **OPG-FC AND RANKL INHIBITORS**

Studies	Study Design	Main outcomes
Teng et al. (2000) [86]	**Animals:** 8-9 week old female mice. **Disease model:** Oral inoculation infection model with *A. actinomycetemcomitans*. **Treatment:** Intraperitoneal injections every other day with PBS or OPG-Fc (1 mg/kg) between weeks 4 and 8.	Reduced alveolar bone loss, decrease the number of osteoclasts.
Mahamed et al. (2005) [87]	**Animals:** 200 NOD mice and 18 BALB/c mice 4-6 weeks old female. **Disease model:** NOD mice were injected with STZ to induce hyperglycemia (40-50 mg/kg). Oral inoculation of *A. actinomycetemcomitans* (10µg/ml). **Treatment:** Intraperitoneal injections with 2.5 µg hu-OPG-Fc / 100 µl PBS, 3 times a week for 8 weeks.	Reduced alveolar bone loss. It showed that RANKL-RANK signaling is involved in controlling increased alveolar bone loss in the local environment.
Jin et al. (2007) [25]	**Animals:** 32 male Sprague-Dawley rats. **Disease model:** Ligature-induced PD placed bilaterally between the lower first molars. **Treatment:** Human OPG-Fc (10 mg/kg) or vehicle by subcutaneous injection twice weekly for 6 weeks.	OPG-Fc suppressed the surface area of osteoclasts in the alveolar crest. Preservation of alveolar bone volume.
Kuritani et al. (2018) [88]	**Animals:** 8 week old male C57BL/6j mice. **Disease model:** LPS-induced calvarial bone destruction.Model of experimental PD using ligatures. **Treatment**: Administration of saline solution, anti-RANKL antibodies (3 mg / kg) or zoledronate (0.2 mg / kg).	Anti-RANKL antibodies significantly inhibited alveolar bone destruction and tooth root exposure. Zoledronate suppressed alveolar bone destruction.

Strontium ranelate (SR)

SR, an antiresorptive compound mainly used for osteoporosis treatment, is a silver-white and soft metallic chemical element. It is placed primarily in areas where mineralization of new bone occurs, such as regions experiencing intramembranous or endochondral ossification [96]. SR is located in the periodic table right down the calcium, and for this reason, it possesses structural and functional similarity to the bone tissue. SR is known as a

divalent cation that has atomic and ionic properties related to calcium and is also considered as a dual-acting agent that diminishes bone resorption by decreasing osteoclastic activity and stimulating bone formation by replication of preosteoblast, and secondarily increasing the activity of functional cells and synthesis of bone matrix and collagen [97, 98]. This dual-acting mechanism of SR (concomitant anti-resorptive and osteoanabolic dual biological activity) represents an advantage over bisphosphonates. Thus, SR is able to increase biomechanical and structural properties of bone, such as mineral density [99]. There are two possible mechanisms of action presented in literature about SR: 1) activating calcium-sensing receptor or another cation-sensing receptor, and 2) increasing expression of OPG in addition to decreasing RANKL expression by osteoblasts [100].

One of the first studies investigating the efficacy of SR in preventing bone resorption was made by Marie et al. [101]. This study tested low SR doses on bone loss induced by estrogen deficiency in female rats. Treatment for 60 days with SR resulted in a dose-dependent increase in plasma, urine, and bone strontium concentrations without any deleterious effect on total or skeletal growth. Furthermore, treatment of OVX rats with SR prevented bone loss and bone mineral content was restored to the values in sham rats. Moreover, SR treatment increased the trabecular bone volume up to 30%. On the other hand, two other studies showed that SR administration did not counteract the loss in bone architecture and bone strength in ovariectomized rats [102, 103]. These contradictory findings lead to a deeper investigation of the potential role of SR in other inflammatory diseases such as PD.

In this context, Karakan et al. [26] investigated the effects of SR administration in rats with ligature-induced PD. Three different dosages of SR were used: 300, 625 and 900 mg/kg and the administration was performed daily by oral gavage. The rats were euthanized 11 days after ligature placement. The results indicated that SR leads to decreased bone loss and reduced osteoclast number. In addition, the number of osteoblast cells was significantly increased after SR treatment. Collectively, the findings of this study suggested that SR at 900 mg/kg may prevent alveolar bone loss in this animal model. Another study conducted by Souza et al. [104] has determined the effect of SR on ligature-induced bone loss in rats. The authors showed that SR prevented periodontal bone loss with concomitant up-regulation of heme oxygenase 1 mRNA levels. A recent study also demonstrated the beneficial effects of SR on alveolar bone loss in rats with concomitant PD and estrogen deficiency [42]. The results indicated that SR prevented ligature-induced bone loss in an estrogen-deficiency

condition and, to a certain extent, increased trabecular bone area in the presence and absenceof periodontal collapse. Furthermore, SR also affected the expression of bone markers (TRAP, RANKL and osteocalcin), appearing to have acted predominantly as an anti- resorptive agent. Taken together, the results of these investigations demonstrated that SR plays an important role in inhibiting bone loss in experimental PD (Table 4).

Table 4. **STRONTIUM RANELATE (SR)**

Studies	Study Design	Main outcomes
Marie et al. (1993) [97]	**Animals:** 112 3-month-old Sprague-Dawley female rats. **Disease model:** Estrogen deficiency-induced bone loss. **Treatment:** 17 beta-estradiol (10ug/kg/day, sc) or divalent strontium by gavage at a dose of 77, 154 or 308 mg/kg/ day or vehicle for 60 days.	Prevented bone loss, and increased trabecular bone volume.
Karakan et al. (2017) [26]	**Animals:** 40 Wistar rats. **Disease model:** Ligature-induced experimental PD placed around the first molars in the right mandible. **Treatment:** Strontium in dosages: 300, 625 and 900 mg / kg. Administration by oral gavage for 11 days.	Less alveolar bone loss, reduced number of osteoclasts, and increased number of osteoblast cells. Best results at a dosage of 900 mg / kg.
Souza et al. (2018) [100]	**Animals:** 48 male Wistar rats. **Disease model:** Ligature-induced PD placed around the upper molars. **Treatment:** Orally administration of strontium ranelate (20 or 100 mg/kg) for 7 days.	Prevented bone resorption and increased heme oxygenase-1 mRNA levels in gingival tissues.
Marins et al. (2020) [42]	**Animals:** 96 female Wistar rats ovariectomized. **Disease model:** Ligature-induced PD in the mandibular first molar. **Treatment:** Oral gavage of 625 mg/kg/day strontium ranelate for 10, 20 and 30 days.	Inhibited bone loss, increased the area of trabecular bone, affected the expression of bone markers.

Biological therapies

Biological therapies are a novel class of compounds mainly used to treat autoimmune diseases such as rheumatoid arthritis and other chronic inflammatory conditions, i.e., Crohn's disease, ankylosing spondylitis and ulcerative colitis [17]. Biological therapies include a range of anti-cytokine agents, including anti-TNF-a, anti-IL-6, anti-IL-1, T and B cells. These specific agents are monoclonal antibodies that act blocking the activity of cytokines and thus inhibiting the immune-inflammatory response of the host, functioning as an immune suppressant [17]. The use of biological agents to manage experimental PD in

animal models has demonstrated potential efficacy for anti-cytokine therapies in ameliorating bone destruction and reducing inflammatory cell infiltrate [105-107], as described below (Table 5).

Table 5. ANTI IL-6 AND ANTI TNF-α

Studies	Study Design	Main outcomes
Apolinário Vieira et al. (2021) [103]	**Animals:** 90 10- to 12-week old male Wistar SPF rats. **Disease model:** PD induced by cotton ligature placed on the right first molar in the mandible. **Treatment:** Systemic administration of Tocilizumab (TCZ) intraperitoneally at concentration dosages (2 mg/kg, 4 mg/kg and 8 mg/kg) for 7 and 14 days.	Inhibited alveolar bone resorption and attachment loss, lower expression of inflammatory infiltrate and lower production of Th17 and RANKL-related cytokines.
Grauballe et al. (2015) [104]	**Animals:** 80 4-week-old obese diabetic male Zucker rats. **Disease model:** PD induced by ligature placement around the maxillary second molars. **Treatment:** Anti-TNF-α Etanercept injections for 5 weeks.	Blocking TNF-α improves metabolic state in obese rats with PD and diminishes periodontal tissue destruction associated with diabetes.
Grauballe et al. (2017) [105]	**Animals:** 52 4-week-old male Zucker rats. **Disease model:** 45 obese rats with type II diabetes and 17 lean rats as controls. PD induced by ligatures around the maxillary second molars. **Treatment:** Treatment with subcutaneous injections of 0.5 ml of 0.78 mg/ml Etanercept or RAGE (Intraperitoneal injections of 0.8 ml of 1.25 g/l ARA) 3 times a week for 5 weeks.	Anti-TNF-α treatment has a positive impact on the subgingival microbial profile in rats with diabetes and ligature-induced bone loss.
Queiroz-Junior et al. (2013) [106]	**Animals:** 40 C57BL6 male mice of 6 week of age **Disease model:** Mice underwent antigen-induced chronic arthritis (AIA). **Treatment**: Intraperitoneal administration of pentoxifylline (50mg / kg) daily for 14 days.	Decreased expression of TNF-α and increased the expression of IL-10 in the maxilla of mice. It did not affect the expression of IFN-γ and IL-17. Decreased joint inflammation.
Oates et al. (2002) [102]	**Animals:** 6 Macaca fascicularis from 3 to 7 years old. **Disease model:** PD induced by silk ligatures inoculated with *P. gingivalis* in lower premolars, first and second molars. **Treatment:** Intrapapillary injections of soluble receptors (blockers), IL-1 and TNF-α (6.6 mg) 3 times a week for 6 weeks.	Reduced radiographic bone loss.

Anti IL-6

A recent study has investigated the effects of systemic administration of anti-IL-6 monoclonal antibodies in the progression of experimental PD in rats [107]. Tocilizumab was intraperitoneally injected immediately after ligature placement and the animals were sacrificed after 7 and 14 days postoperatively. The results indicated that tocilizumab diminished alveolar bone resorption and attachment loss. Moreover, inflammatory infiltrate

was also decreased after treatment. The authors suggested that modulatory therapy with biological agents might be an interesting alternative to inhibit alveolar bone loss, and further studies are warranted to confirm the data.

Anti TNF-α

Tumor necrosis factor-alpha is a key signaling modulator in the pathogenesis of PD and its up-regulation is associated with increased osteoclastogenesis. Thus, investigations targeting TNF-α have been evaluated to manage inflammatory bone resorption in animal models. In this context, a recent study evaluated the effects of systemic administration of Etanercept in mice with concomitant diabetes melitus and periodontitis [108]. Obese diabetic zucker rats were systemically administered with Etanercept and one week later received ligature to induce experimental PD. Animals were sacrificed after 5 weeks from the baseline. This study indicates that blocking TNF-α improves the metabolic status in obese rats with PD and decreases periodontal breakdown associated with diabetes. The same research group also confirmed that anti-TNF-α treatment positively impacts the subgingival microbial profile in rats with diabetes and ligature-induced bone loss [109]. Another study investigated anti-TNF-α effects with pentoxifylline in an experimental mouse model of chronic antigen-induced arthritis (AIA) associated PD [110]. The authors demonstrated that the treatment employed was able to diminish joint inflammation, reduce the levels of TNF-α and IL-17, and prevent signs of PD (decreased the number of osteoclasts and recruitment of neutrophils in the connective tissue). In addition, the treatment employed showed the anti-inflammatory and bone protective effects in mice with AIA and concomitant PD. Accordingly, a previous study also demonstrated the positive effects of anti-TNF-α on the progression of experimental PD induced by ligature placement by decreasing radiographical bone loss [106]. Finally, Cirelli et al in 2009 have used adeno-associated virus vector based on serotype 1 (AAV2/1) to deliver the TNF receptor-immunoglobulin Fc (TNFR:Fc) fusion gene to rats subjected to experimental periodontitis by means of *P. gingivalis* LPS-mediated bone loss [111]. The results showed that AAV2/1-TNFR:Fc administration diminished the levels of several proinflammatory cytokines and osteoclast-like cells in the connective tissue of rats. These data indicate that delivery of AAV2/1-TNFR:Fc might be a feasible approach to modulate PD progression.

Herbal medicine

Curcumin

Curcumin is a bioactive compound of turmeric and derived from *curcuma longa*, a tropical plant native to Southeast Asia [112]. It is a yellow hydrophobic polyphenol composed of three curcuminoids, and it is largely used in dietary spice. It has been reported that curcumin has a variety of biological activities, including osteo-immune modulatory properties, anti-inflammatory, antioxidant, anti-angiogenic and anti-bacterial effects with the capacity to modulate the innate immune host response [30, 45, 113-116]. Due to the innumerous beneficial effects described in the literature with the use of curcumin to treat experimental PD, natural or chemically modified curcumin has been suggested as an interesting therapeutic approach to managing inflammatory bone resorption [29, 30, 44, 45, 113-116]. Nevertheless, different variables, such as diverse dosages (in vitro and in vivo), vehicle used, and administration route (intraperitoneally, intravenously, and orally) have also been described in the literature [29, 30, 45, 114, 116, 117].

Many investigations have been carried out to evaluate curcumin effects during the progression of experimental PD in murine [29, 30, 44, 45, 113-117] (Table 6). Recently, Pimentel et al. [118] assessed the impact of curcumin (100 mg/kg) on the progression of experimental PD in diabetic rats. The PD model was induced by placing cotton ligatures around the first and second maxillary molars and an injection of streptozotocin-induced diabetes. Curcumin was administered daily by oral gavage for 30 days. The results indicated that natural curcumin reduces alveolar bone loss and favorably modulates the osteo-immune inflammatory process during disease progression. Interestingly, Zambrano et al. [30] investigate the local administration of curcumin-loaded nanoparticles in an experimental PD model. A model of *Escherichia coli* bacterial lipopolysaccharide (LPS) injection was used to induce PD. The curcumin nanoparticles were locally injected, 2 times per week for four weeks, in the palatal mucosa around the first maxillary molar. Radiographical analysis (micro-CT) significantly reduced inflammatory bone resorption and osteoclast counts. A previous study [119] using the silk ligature model of PD in rats demonstrated the potent capacity of oral administration of curcumin (100mg/kg/day) for 30 days to inhibit bone resorption, which is in agreement with the above-reported studies [30, 118].

Table 6.	CURCUMIN	
Studies	**Study Design**	**Main outcomes**
Pimentel et al. (2020) [114]	**Animals:** 100 10-week-old male rats. **Disease model:** Diabetes was induced by streptozotocin. PD induced by ligatures in the lower first molar and upper second molar. **Treatment:** Curcumin (100mg/kg) and placebo solutions and insulin administration by gavage for 30 days.	Decreased linear bone loss in the molar region. Reduced RANKL / OPG ratio.
Zambrano et al. (2018) [30]	**Animals:** 16 Holtzman rats. **Disease model:** PD induced by injections of 3 µL LPS (10 mg/mL from *E. coli*) into the maxillary tissues. **Treatment:** 3ul of nanocurcumin was injected contralaterally from the left side into the gingival tissues twice a week.	Inhibition of inflammatory bone resorption and decreased osteoclast count and inflammatory infiltrate; marked attenuation of p38 MAPK and NF-kB activation.
Corrêa et al. (2017) [115]	**Animals:** 40 Wistar rats. **Disease model:** Periodontitis induced by silk ligatures around the first molars. **Treatment:** Administration by gavage of placebo solution, 10 mg/kg resveratrol, 100 mg/kg curcumin or 10 mg/kg resveratrol plus 100 mg/kg curcumin for 30 days.	Diminished bone loss and inflammatory infiltrate for resveratrol + curcumin group.
Almeida Brandão et al. (2019) [109]	**Animals:** 35 male albino rats **Disease model:** PD induced by LPS injections (*E. coli*) in gingival tissues. **Treatment:** Oral gavage of chemically modified curcumin (CMC2.24) at doses: 1, 3, 10 and 30 µM for 28 days.	Inhibited alveolar bone resorption, osteoclastogenesis and expression of TNF-α, regardless of doseges.
Curylofo-Zotti et al. (2018) [45]	**Animals:** 50 male rats. **Disease model:** PD induced by LPS injections into the gingival tissues in the maxilla three times a week. **Treatment:** 2% CMC, CMC2.24 30 mg/ kg, Curcumin 100 mg/kg. Administered by gavage for 15 days.	CMC2.24 was able to reduce alveolar bone resorption.
Elburki et al. (2017) [117]	**Animals:** 18 male Sprague-Dawley rats. **Disease model:** Diabetes induced by intravenous injection of streptozotocin. PD induced by LPS injection into the maxilla. **Treatment:** CMC 2.24 daily administered by oral gavage (30 mg/kg) for 3 weeks.	It inhibited alveolar bone loss and local and systemic inflammation.
Elburki et al. (2014) [110]	**Animals:** 11 male Holtzman rats. **Disease model:** PD induced by LPS injection into the gingival tissue in the maxilla three times a week for 14 days. **Treatment:** Daily oral administration of CMC 2.24 (30 mg/kg) for 14 days.	Less alveolar bone loss, inhibited inflammation and destructive collagen mediators.

Previous studies have used different strategies to enhance the clinical application of curcumin to treat experimental PD. Indeed, chemically modified compounds have been developed to increase their clinical efficacy, which resulted in greater bioavailability maintaining its biological and safety properties [45, 113, 114, 120]. De Almeida Brandao et al. [113] evaluated the effects of a modified curcumin so-called CMC22.4 that is a novel bis-

dimethoxy-4-phenylaminocarbonyl curcuminoid. In this study, rats underwent experimental PD using direct microinjections of *Escherichia coli* bacterial LPS into the gingival tissue around the first maxillary molars three times per week. Curcumin was administered daily by oral gavage immediately after LPS injection and continued for the whole experimental period of 28 days. The outcomes showed that CMC2.24 inhibited bone loss, inflammation and osteoclastogenesis in the LPS-induced periodontitis model even at a low dosage (1 mg/kg/day), suggesting that this compound is more effective than previously documented. Curylofo-Zotti et al. [45] also investigated the effects of CMC2.24 in a model of LPS-induced PD. Similar to the study mentioned above [113], the authors showed that oral administration of curcumin CMC2.24 (30 mg/kg/day) significantly inhibited inflammatory infiltrate in the gingival tissue, decreased the number of osteoclasts and abrogates bone resorption, pointing to an interesting potential of CMC2.24 in prevent bone resorption in an inflammatory model of PD. Similarly, Elburki et al. [121] showed that oral gavage with CMC2.24 (30 mg/kg/day) also reduced inflammation-mediated connective tissue breakdown in rats with diabetes (induced by intravenous injection of streptozotocin) and PD (induced by *E. coli* LPS injections) and prevented hyperglycemia-induced tissue destruction. CMC2.24 was also able to attenuate the severity of inflammation and bone loss in the periodontal tissues, acting as a potential therapeutic inhibitor of bone resorption in inflammatory conditions. These findings parallel previous observations by the same research group [114] that demonstrated the positive effects of CMC2.24 in inhibiting bone resorption during LPS-induced experimental PD in rats.

Taken together, several studies have demonstrated the beneficial effects of natural curcumin or chemically modified curcumin to treat experimental PD without adverse side effects. Nevertheless, it is important to bear in mind that the differences in dosages used in the studies, the low absorption rate, reduced half-life, and rapid systemic elimination [122] might limit its clinical use to treat PD in humans.

Chalcones

Chalcone is a medicinal plant that has been conventionally used in Brazilian medicine to treat bleeding gums [123]. It is a phenolic compound extracted from the *Myracrodruon urundeuva* (Engl.). This compound presents analgesic and anti-inflammatory properties as evidenced by previous studies in experimental models of inflammation [123, 124]. Moreover, antioxidant, antimicrobial and antiresorptive properties were previously described during inflammatory conditions, including RA and inflammatory bowel diseases [125, 126]. Therefore, based on the assumption that chalcone presents beneficial properties in inflammation, previous studies have investigated its potential therapeutic effects during experimental periodontitis in rats.

In a study of ligature-induced periodontal bone loss in rats, Botelho et al. [127] assessed the effects of a gel containing chalcones during the progression of PD. Rats underwent nylon ligature placement around the second maxillary molars and received immediately after its placement the chalcone gel (600 µg/g gel) topically applied to the gingival tissues three times per day during the entire experimental period (11 days). The results showed that chalcone gel prevents alveolar bone resorption in the conditions studied and presented with anti-inflammatory and antimicrobial effects during the course of PD.

More recently, Fernandes et al. [46] evaluated the effects of chalcone T4 during the progression of experimental PD. In this study, PD was induced by placing a cotton ligature around the first mandibular molar. Chalcone T4 was systemically administered daily by intragastric gavage (5 and 50 mg/kg) starting on the same day of ligature placement. After 15 days of treatment, the animals were sacrificed, and measurements of radiographical, histological and molecular analyses were performed. The data indicate that 5mg/kg of chalcone T4 decreased bone resorption and cellular infiltrate in the connective tissue. Moreover, in vitro data demonstrated that this treatment resulted in a reduced number of osteoclasts and resorption area in raw 267.4 cells. As a proof-of-concept study, the data suggested the potential effect of chalcone T4 as an adjuvant for experimental PD treatment. More studies are warranted to investigate dose-response, the effects in different inflammatory models, and the factors that might influence its bioavailability, to better comprehend the pharmacokinetics and pharmacodynamics behavior of Chalcone T4 [46].

Flavonoids

In an attempt to pursue natural products with pharmacokinetic, anti-inflammatory, antioxidant, and immunomodulatory effects, growing attention has been dedicated to searching phenolic compounds that might have protective effects on bone and connective tissue [128]. Flavonoids, a group of polyphenolic compounds found in many plants (soybean, olive), fruits (orange peel), vegetables, seeds and beverages have been suggested as a possible alternative to treat inflammatory bone resorption due to its wide range of biological properties and activities [129]. Therefore, the dietary intake of natural ingredients, including innumerous flavonoids, might be beneficial for bone tissues and can prevent PD progression and severity in different animal models of periodontitis. In this context, many studies have used different types of flavonoids to prevent and treat experimental periodontitis with beneficial effects on the alveolar bone tissue without adverse effects [31-33, 128, 130-135].

Genistein, an isoflavone found in soybean, attenuates alveolar bone loss in a rat model of ligature-induced periodontitis [132]. It has also been reported that genistein inhibits bone loss in ovariectomized (OVX) mice, pointing to an important role in preventing experimental postmenopausal osteoporosis [136]. Taxofolin is a flavanone with potent antioxidant properties that has been shown to stimulate osteoblast differentiation and suppress osteoclastogenesis in vitro [137]. Recently, Alpan et al. [135] demonstrated that taxofolin attenuates inflammatory bone resorption in a model of ligature-induced bone loss in rats, decreases inflammatory infiltrate, and improves alveolar bone formation. In an experimental model of LPS-induced inflammatory bone loss, the administration of the flavonoids nobiletin and tangeretin was able to suppress LPS-induced osteoclast formation and bone loss. Furthermore, both flavonoids inhibited osteoclastogenesis in RAW264.7 macrophages [138]. Similarly, the effect of a flavonoid from the bergamot juice could inhibit bone loss and decrease gingival inflammation markers in a rat model of LPS-induced PD [133]. Huang et al. [134] evaluated the effects of myrcetin, a naturally occurring flavonoid compound, in an experimental OVX mouse PD model. Systemic administration of myrcetin prevented bone loss and enhanced alveolar crest height in vivo, and attenuated osteoclast formation and bone resorption in vitro [134] (Table 7).

Table 7.	FLAVONOIDS	
Studies	**Study Design**	**Main outcomes**
Alpan et al. (2020) [131]	**Animals:** 32 male Wistar rats. **Disease model:** PD induced by ligatures in the lower first molars. **Treatment:** Administration by oral gavage of taxifolin at doses: 1 mg/kg and 10 mg/kg for 29 days.	Reduced alveolar bone loss. High BMP-2, OCN, ALP and Col 1 expression; and lower RANKL immunoexpression.
Tominari et al. (2012) [134]	**Animals:** 6-week-old male mice. **Disease model:** LPS-induced bone loss (25 μg) on days 0, 2 and 4 for 7 days. **Treatment:** Flavonoids - Nobiletin or Tangeretin (30 μM) for 7 days.	Both flavonoids suppressed osteoclast formation and bone resorption. Decreased osteoclastogenesis in RAW264.7 macrophages.
Gugliandolo et al. (2019) [129]	**Animals:** 40 male Sprague-Dawley rats. **Disease model:** PD induced by LPS injection (10 μg/μl) in the gingival tissue between the first and second molars. **Treatment:** Bergamot juice flavonoids, 20 mg/kg administered by oral gavage for 14 days.	Flavonoid improved the inflammatory process in the gingival tissues. Decreased NF-κB activation, and pro-inflammatory cytokine levels.
Huang et al. (2016) [130]	**Animals:** 24 8 weeks old ovariectomized female C57BL/6 mice. **Disease model:** Ligature-induced PD in maxillary molars. **Treatment:** Intraperitoneal injections of low or high dose myricetin (2 or 5 mg) every other day for 30 days.	In vivo, it suppressed bone loss, increased alveolar crest height. In vitro, it inhibited osteoclast formation and bone resorption.
Cheng et al. (2010) [127]	**Animals:** 6-week-old male Sprague-Dawley rats. **Disease model:** Ligature-induced PD in the molars of the maxilla and mandible. **Treatment:** Quercetin (75 mg/kg) for 5 days. LPS (5 mg/mL) and quercetin plus LPS.	Decreased alveolar bone loss and reduced inflammatory cell infiltrate in connective tissue. Decreased LPS-induced osteoclast formation in vitro.
Carvalho et al. (2021) [126]	**Animals:** 60 BALB/c 4-week-old male mice. **Disease model:** PD induced by microinjections of LPS on the palatal surface of both first molars. **Treatment:** Food supplement of eriocitrin and eriodictiol (25 and 50 mg) for 30 days.	Inhibited periodontal inflammation.
Kuo et al. (2019) [33]	**Animals:** 48 male rats. **Disease model:** Ligature-induced PD in upper and lower first second molars. **Treatment:** Hesperidin at doses 75 or 150 mg/kg by intragastric gavage for 7 days.	Inhibited alveolar bone loss and the production of pro-inflammatory mediators.
Balci Yuce et al. (2019) [31]	**Animals:** 28 male Wistar rats. **Disease model:** Ligature-induced PD around the lower right first molars. **Treatment:** Luteolin 50mg or 100mg given by oral gavage for 11 days.	Decreased bone loss in both groups. Greater number of osteoblast cells and decreased number of inflammatory cells.

Taskan et al. (2019) [124]	**Animals:** 32 female Wistar rats. **Disease model:** Ligature-induced PD in the lower right first molar. **Treatment:** Administration by oral gavage of Oleuropein 12 or 25 mg / kg for 14 days.	Decreased alveolar bone loss due to decreased osteoclastic activity, inflammation and apoptosis and increased osteoblastic activity.

Quercetin is an abundant flavonol-type flavonoid that has been associated with innumerous beneficial effects regarding the inflammatory process and immune functions [139-141]. The effects of quercetin on the progression of experimental PD were evaluated by Cheng et al. [131]. Utilizing a model of ligature-induced bone loss, the authors demonstrated decreased alveolar bone loss and reduced inflammatory cell-infiltrate in the connective tissue of rats that have received systemic administration of quercetin. Moreover, in vitro data demonstrated that quercetin diminished LPS-induced osteoclast formation, suggesting that it might possess an ameliorative effect during PD progression [131]. Recently, it was demonstrated that a citrus flavonoid - eriocitrin and eriodicytol - diminished inflammatory cell infiltration in the connective tissue of rats with induced PD by means of LPS-injections suggesting that a diet supplemented with flavonoids might enhance local immunity and host defense [130]. Finally, other studies showed beneficial effects of hesperidin [33], luteolin [31], and oluropein [128] on alveolar bone loss and inflammation in a rat model of ligature-induced PD indicating that flavonoids might be an interesting candidate for modulating inflammatory disease.

Specialized Proresolving Mediators (SPM)

Current key discoveries in the mechanisms of inflammation during PD initiation and progression encouraged the search for new treatment alternatives for PD using proresolving mediators. Resolution of inflammation comprises active biochemical programs that allow inflamed tissue to return to homeostasis [142, 143]. SPMs are a novel family of oxylipids mediators, including resolvins, maresins, lipoxins and protectins derived from omega-3 polyunsaturated fatty acid (PUFA), which regulate the inflammatory process without immunosuppression [6]. The SPMs function in inflammation termination by activating specific mechanisms to restore tissue homeostasis [142, 143]. Briefly, they selectively inhibit leukocyte recruitment, activate macrophage phagocytosis of microorganisms, stimulate infiltration of monocytes, and stimulate the expression of molecules involved in antimicrobial defense [144]. Such SPMs promote tissue repair, eliminate bacteria, increase

the host defense, and impact the responses of adaptive immune cells (Figure 3) [38]. The E-series resolvins (RvE1) are biosynthesized from the eicosapentaenoic acid (EPA), and it is considered a stereoselective agonist that interacts with two identified G protein-coupled receptors: BLT1 (expressed on neutrophils) and chemerin receptor 23 (chemR23) expressed on macrophages, monocytes, dendritic cells and osteoblasts [145, 146]. RvE1 interacts with BLT1 or chemR23 to inhibit leukocyte infiltration and cytokine production, thus promoting the resolution of inflammation [144].

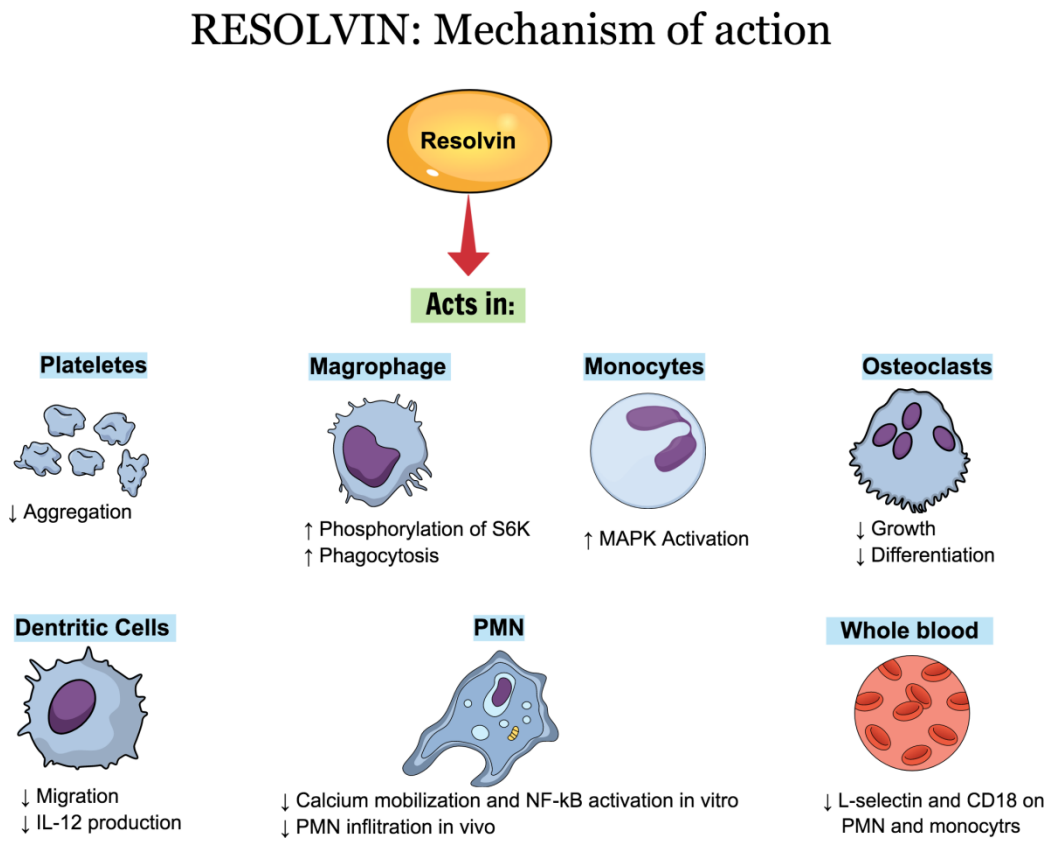

Fig. 3 - Resolvins (RvE1) act to reduce ADP-stimulated platelet aggregation. In macrophages, RvE1 increases S6K (ribosomal protein S6 kinase) phosphorylation and phagocytosis. In monocytes, MAPK (mitogen-activated protein kinase) activation occurs. RvE1 acts on osteoclasts decreasing their growth and differentiation. In dendritic cells, there is a decrease in their migration and in the production of IL-12. In vitro RvE1 reduced calcium mobilization and activation of NF-kB and in vivo there was a lower infiltration of PMN (polymorphonuclear cell/neutrophil). In the blood, there is a decrease in L-selectin and CD18 in PMN and monocytes.

SPM show significant effectiveness in treating inflammatory conditions including inflammatory pain [147], experimental PD [39, 148, 149], and bone preservation [41]. Furthermore, it has been reported that SPM attenuates atherosclerotic plaque formation in diet and inflammation-induced atherogenesis [150]. Gao et al. [41] showed that transgenic mice overexpressing the human chemR23 were able to diminish the destruction of the alveolar bone induced by ligature placement. Moreover, local RvE1 treatment accelerated the regeneration of bone defects in a craniotomy model. Taken together, RvE1 modulates osteoclast differentiation and bone remodeling, rescuing OPG production and restoring a favorable RANKL/OPG ratio [41]. This data agrees with the previous report that evaluated the impact of RvE1 on bone remodeling in mice, using a calvarial osteolytic model with or without systemic administration of RvE1 [151]. The data demonstrated that RvE1 reduced bone resorption and osteoclastogenesis. RvE1 also negatively regulated osteoclast differentiation, which resulted in a reduction in inflammatory bone resorption, suggesting that RvE1 may be a therapeutic potential for treating inflammatory diseases [151]. Lee et al. [149] also demonstrated that topical application of RvE1 downregulated bone loss induced by ligature placement, decreased the inflammatory process and the number and size of osteoclasts in rats. In addition, RvE1 induced changes in the composition of the local microbiota suggesting the modulation of local inflammation has an important role in forming the subgingival microbiota composition [149].

Hasturk et al. demonstrated that topical application RvE1 was able to prevent initiation and progression of experimental PD, and even induce the regeneration of periodontal tissues (alveolar bone, periodontal ligament and cement) in a rabbit model of ligature-induced bone loss [39, 148]. RvE1 downregulated the progression of PD by decreasing pro-inflammatory mediators and reducing inflammatory bone loss. Furthermore, RvE1 is able to enhance the clearance of PD-associated bacteria [39, 148]. These outcomes suggest that PD-associated bacteria actively direct the protective bactericidal immune response into a dysfunctional state, which may be reversed by SPMs. The established protective action of SPM aiming in promoting the resolution of inflammation in innumerous animal models of PD makes them an interesting alternative to treat PD [6, 152]. Table 8 depicts the primary findings of the selected studies.

Table 8. SPECIALIZED MEDIATORS IN PRO-RESOLUTION (SPM)		
Studies	**Study Design**	**Main outcomes**
Gao et al. (2013)[41]	**Animals:** chemR23tg mice. **Disease model:** Ligature-induced PD in the upper left second molar; 1 mm craniotomy defect. **Treatment:** RvE1 (100ng in 20 µl PBS) or vehicle was injected subcutaneously during craniotomy every 2 days.	Less destruction of the alveolar bone after ligatures. It accelerated bone defect regeneration in a craniotomy model.
Lee et al. (2016) [145]	**Animals:** 18 6-week-old male Wistar rats. **Disease model:** Ligature-induced PD placed on the upper right and left second molars. **Treatment:** Topically Resolvin E1 at 0.28 mM or 1.4 mM, 3 times a week for 4 weeks.	It reversed bone loss and inflammatory gene expression and reduced osteoclast number for both dosages.
Hasturk et al. (2006) [39]	**Animals:** 21 male white rabbits. **Disease model:** Ligature-induced PD folowed by *P. gingivalis* injection around the second mandibular premolars. **Treatment:** RvE1, 4 µg applied every other day for 6 weeks.	Less progression of PD, decreased pro-inflammatory mediators and reduced inflammatory bone loss.
Hasturk et al. (2007) (144]	**Animals:** 39 male white rabbits. **Disease model:** Ligature-induced PD followed by *P. gingivalis* infection around the second mandibular premolar **Treatment:** RvE1, 4 µg applied every other day for 6 weeks.	Hard and soft tissue regeneration and decreased inflammation in the periodontal tissues.

Probiotics

The manipulation of the intestinal microbiota through probiotics has been proposed to alter bone remodeling during the course of PD both in pre-clinical studies and in randomized clinical trials. The rationale for this approach is based on the concept that bone health is affected by changes in the intestinal microbiota and therefore, strategies to induce beneficial effects through nutritional supplementation with probiotics have been evidenced. The term probiotics were introduced by Lilly and Stillwell in 1965 [153]. Probiotics are live microorganisms that, when administered in adequate amounts, confer beneficial effects on the host's health. They repopulate beneficial bacteria, which can help kill pathogenic bacteria and fight infection. Orally administered probiotics can benefit oral health by preventing microbiota growth or modulating mucosal immunity in the oral cavity [154]. Probiotics can help prevent and treat PD through several mechanisms, including direct interaction, competitive exclusion, and modulation of the host's immune response. Studies show that the

treatment strategies conferred by probiotics against PD occur mainly by inhibiting specificpathogens or altering the host's immune response [155] (Table 9).

Table 9.	PROBIOTICS	
Studies	**Study Design**	**Main outcomes**
Moraes et al. (2020) [152]	**Animals:** 32 male rats. **Disease model:** Ligature-induced PD; ligature and live *L. reuteri*; ligature and dead *L. reuteri*, in the lower first molars. **Treatment:** Live or dead *L. reuteri* given orally 30 days before the disease and 14 days after.	Increased alveolar bone volume and trabecular number.
Cardoso et al. (2020) [153]	**Animals:** 32 male Wistar rats. **Disease model:** Ligature-induced PD and. CIA arthritis model. **Treatment:** Probiotic (HN019) in deionized water was supplied to animals (1.5×10^9 CFU/ml) for 39 days.	Reduced alveolar bone loss, TNF-α and IL-6 levels and increased IL-17 levels. Decreased levels of ACPA antibodies.
Ricoldi et al. (2017) [154]	**Animals:** 32 adult male Wistar rats. **Disease model:** Ligature-induced PD around the lower right first molars. **Treatment:** 10 ml of 10% skim milk with *B. Lactis* HN019 once daily for 15 days.	Reduced alveolar bone resorption and attachment loss. Increased expression of anti-inflammatory cytokines and reduced expression of pro-inflammatory cytokines.
Oliveira et al. (2017) [155]	**Animals:** 32 adult male Wistar rats. **Disease model:** PD induced by cotton ligatures around the lower first molars. **Treatment:** Probiotic HN019 administered topically to the subgingival region of molars on days 0, 3 and 7.	Less alveolar bone resorption and attachment loss.
Gatej et al. (2018) [156]	**Animals:** 36 6-week old BALB/c mice. **Disease model**: PD was induced by oral inoculation with *P. gingivalis*. **Treatment:** Probiotic Lactobacillus rhamnosus was given by oral gavage before and during disease induction.	Reduced bone loss and gingival inflammation.
Maekawa et al. (2014) [157]	**Animals:** C57BL male mice **Disease model**: Ligature-induced PD around the upper left second molar. **Treatment:** *L. brevis* CD2 applied topically between the gingiva and the buccal mucosa.	Decreased bone loss and lower expression of TNF, IL-1β, IL-6 and IL-17A.
Kobayashi et al. (2017) [158]	**Animals:** 36 8-week-old BALB/c mice. **Disease model:** PD induced by injection of *P. gingivalis* in the mandibular molars. **Treatment:** *Lactobacillus gasseri* SBT2055 (LG2055) given by gavage daily for 5 weeks.	Reduced alveolar bone loss and decreased TNF-α and IL-6 expression in the gingival tissue.
Levi et al. (2018) [159]	**Animals:** 40 male Wistar rats. **Disease model:** Ligature-induced PD **Treatment:** *Mannanoligosaccharide* (MOS) added daily to the food for 30 days prior to PD.	Decreased alveolar bone loss, and increased bone mineral density. Decreased expression of IL-10 and IFN-γ and TNF-α genes.

Several studies have been published using probiotics for the treatment of experimental PD. Moraes et al. investigated the effects of *L. reuteri* administration during the development of induced PD in rats [156]. The results showed that treatment with probiotics increased the percentage of bone volume and the thickness and number of trabeculae and decreased bone porosity and trabecular separation. Cardoso et al. evaluated the effects of systemic administration of the probiotic *Bifidobacterium animalis* HN019 on ligature-induced periodontitis in rats with experimental RA [157]. Probiotic treatment in animals with experimental arthritis and PD reduced alveolar bone loss, TNF-α and IL-6 levels, and increased IL-17 levels compared to those without probiotics. Furthermore, there was a decrease in the levels of anti-citrullinated protein antibodies in animals with experimental RA. Ricoldi et al. [158] and Oliveira et al. [159] found similar results using HN019 to treat experimental PD, showing reductions in alveolar bone resorption and connective tissue attachment loss. These results were also observed using different strains of probiotics, including *Lactobacillus rhamnosus* [160], *Lactobacillus brevis* CD2 [161] and *Lactobacillus gasseri* SBT2055 [162].

Some limitations associated with the use of probiotic therapy (difficulty of exogenously administered bacteria in remaining in the oral environment) have stimulated the search for other strategies capable of manipulating the ecology of the oral biofilm [163]. An interesting approach concerns the nutritional stimulation of beneficial native bacteria to promote oral health. Prebiotics favor changes in microbial composition or activity, aiming to stimulate the growth of health-promoting bacteria in the resident intestinal microbiota, which provides local and systemic benefits for the host's health. By definition, prebiotics are selectively fermented ingredients that allow specific changes, either in the composition and/or activity of the gastrointestinal tract microflora, that confer benefits to the host, well-being and health. They are substances not digested by enzymes, salts and acids produced by the body. Currently, only oligosaccharides (fructooligosaccharides and galactooligosaccharides) can be called prebiotics. Their mechanism of action occurs through: a) improvement in the growth of resident commensal intestinal bacteria, particularly *bifidobacteria* and *lactobacilli*; b) They exert a direct effect on the host by stimulating the expression of IL-10 and INF-g, increased secretion of immunoglobulin (IgA) and modulation of inflammatory responses in pathogens [163].

Prebiotics and probiotics often work synergistically and, when combined in the same product, are known as symbiotics. Symbiotics contain both probiotic and prebiotic components. The rationale for such products is that the combination increases the survival of probiotic bacteria in the passage through the proximal region of the gastrointestinal tract, improving colonization of the probiotic in the large intestine, stimulating the effect on the growth of endogenous flora. The main prebiotics evaluated in humans are fructans and galactans. Mannan oligosaccharides (MOS) are also gaining importance. Levi et al. [164] performed a pre-clinical study in rats demonstrating that animals with ligature-induced PD showed changes in intestinal morphology compared to animals without the disease, confirming the possible relationship between oral and intestinal dysbiosis. When animals with experimental PD were treated with MOS, the intestinal morphology became more similar to that of animals without disease, demonstrating prebiotics' protective role in the intestinal environment under conditions of oral dysbiosis. Furthermore, animals with PD and MOS had less severe PD than those not treated with MOS. In fact, recent scientific evidence suggests that manipulating the microbiota through prebiotics and probiotics confers health benefits on the host through different mechanisms, improving periodontal health and other common skeletal diseases such as arthritis and osteoporosis.

Conclusion

This comprehensive review of the literature summarizes the main findings of studies that have used pharmacological drugs to manage experimental PD. The use of modulators of the immune host response or antiresorptive medications offers interesting alternatives to inhibit bone loss and decrease the inflammatory infiltrate in the connective tissue. All those treatments tested can help modulate the host inflammatory response and ameliorate the progression of the experimental disease. As stated earlier, the primary treatment of PD is through a mechanical approach, SRP, to remove the attached biofilm into the tooth and root surface. However, this local treatment does not respond equally well in susceptible patients. Thus, adjunctive therapies that decrease the inflammatory host response plays an important role in achieving better clinical outcomes, especially in patients with associated comorbidities, such as diabetes melitus and rheumatoid arthritis. It is important to bear in mind that some of the included drugs in this review, i.e., bisphosphonate, biological agents, RANKL and CtsK inhibitors possess some side effects that might limit their clinical use. Therefore, herbal medicine and supplementation with omega 3 and probiotics have gained

growing attention due to its modulatory and antiresorptive activities and the lack of side effects being considered promising alternatives as adjunctive to SRP in susceptible patients.

Author Contribution

The authors confirm contribution to the paper as follows: study conception and design: JAC, and RSM; draft manuscript preparation: ALRP, BSM, EBBP, FASM, JAC, and RSM. All authors reviewed the article text and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest to report regarding the present study.

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