UNESP - Universidade Estadual Paulista

“Júlio de Mesquita Filho”

Faculdade de Odontologia de Araraquara

Mariely Araújo de Godoi

Influência da inibição de JAK na progressão da periodontite

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Orientador: Profª. Drª. Morgana Rodrigues Guimarães Stabili

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Mariely Araújo de Godoi

Influência da inibição de JAK na progressão da periodontite

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“Foi o tempo que dedicaste a tua rosa que fez a tua rosa tão importante.”

SAINT-EXUPÉRY [*]

[*] Saint-Exupéry A. O pequeno princípe. 31.ed. Rio de Janeiro: Agir; 1987.

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RESUMO

A periodontite, semelhante a outras doenças crônicas, envolve a ativação de citocinas que são moduladas por vias de sinalização intracelular, incluindo a via Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT). Considerando o papel desta via de sinalização no processo inflamatório, diversos inibidores farmacológicos de JAK foram aprovados pela Food and Drug Administration (FDA) para o tratamento de doenças inflamatórias, e têm sido associados a redução dos sinais e sintomas destas doenças, além de apresentarem um perfil farmacológico seguro e eficaz. Na periodontite, poucos estudos avaliaram o papel de JAK no processo de patogênese da doença, e os resultados são divergentes. O presente estudo tem como objetivo avaliar o efeito da administração sistêmica de inibidores de JAK1-3 e JAK3, sobre a progressão da periodontite em ratos, e investigar seu papel modulatório em células do microambiente periodontal. No estudo in vivo, os animais foram submetidos a colocação de ligaduras nos primeiros molares inferiores para indução da periodontite, e receberam, concomitantemente à progressão da doença, água destilada ou inibidores de JAK1-3 ou JAK3, via oral (gavagem intragástrica) por 7 dias. Animais do grupo controle negativo, sem ligaduras, receberam administração de água pelo mesmo período. Ao fim do período experimental os animais foram eutanasiados, e as mandíbulas contendo tecido gengival ao redor do primeiro molar, removidas para avaliação dos seguintes desfechos: reabsorção óssea alveolar; quantificação do número de osteoclastos; expressão gênica de Il-6, Tnf-alfa e Rankl, e proteica de TNF-alfa; expressão e localização de células inflamatórias (CD45 e CD3); e quantificação de elementos celulares (infiltrado celular, vasos sanguíneos e matriz extracelular). Os resultados demonstraram que ambos os inibidores preveniram a perda óssea induzida pela periodontite ($p<0.05$), e reduziram a quantidade de osteoclastos em quase 30%. Redução significativa do infiltrado celular, bem como um aumento da matriz colágena e de vasos sanguíneos foram encontrados nos animais tratados com os inibidores ($p<0.05$). Corroborando os dados da análise estereométrica, os inibidores de JAK também reduziram a quantidade de células CD45+ (marcador de leucócitos) no tecido gengival. Redução significativa na expressão dos marcadores inflamatórios (em nível gênico e proteico) também foi encontrada no tecido gengival dos animais tratados com os inibidores farmacológicos. Adicionalmente, experimentos in vitro investigaram o efeito dos inibidores de JAK sobre a diferenciação de osteoclastos (RAW 264.7) induzidos por RANKL, produção de espécies reativas de oxigênio (ROS), bem como sobre a formação de nódulos de mineralização e proliferação de células pré-osteoblásticas (MC3T3-E1). Através da microscopia de fluorescência, observamos que ambos os inibidores demonstraram redução significativa na formação de osteoclastos. Os dados também indicaram que a inibição de JAK inibiu a produção de ROS após estímulo com LPS. Em relação à osteogênese, foi observado que a inibição de JAK estimulou a proliferação celular e a formação de nódulos de mineralização. Os resultados demonstram que a inibição das isoformas de JAK apresentaram um impacto significativo na redução das alterações inflamatórias em animais com periodontite, indicando o protagonismo da via na patogênese e progressão da doença, além do papel relevante sobre células do metabolismo ósseo.

Palavras-chave: Periodontite. Inibidores de Janus Quinases. Transdução de sinais. Imunomodulação.

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ABSTRACT

Periodontitis, similar to other chronic diseases, involves the activation of cytokines that are modulated by intracellular signaling pathways, including the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway. Considering the role of this signaling pathway in the inflammatory process, several pharmacological JAK inhibitors have been approved by the Food and Drug Administration (FDA) for the treatment of inflammatory diseases, and have been associated with reducing the signs and symptoms of these diseases, in addition to presenting a safe and effective pharmacological profile. In periodontitis, few studies have evaluated the role of JAK in the disease pathogenesis process, and the results are divergent. The present study aims to evaluate the effect of systemic administration of JAK1-3 and JAK3 inhibitors on the progression of periodontitis in rats, and to investigate their modulatory role in cells of the periodontal microenvironment. In the in vivo study, the animals underwent ligatures placed on the lower first molars to induce periodontitis, and received, concomitantly with disease progression, distilled water or JAK1-3 or JAK3 inhibitors, orally (intragastric gavage). for 7 days. Animals in the negative control group, without ligatures, received water administration for the same period. At the end of the experimental period, the animals were euthanized, and the jaws containing gingival tissue around the first molar were removed to evaluate the following outcomes: alveolar bone resorption; quantification of the number of osteoclasts; gene expression of Il-6, Tnf-alpha and Rankl, and protein of TNF-alpha; expression and localization of inflammatory cells (CD45 and CD3); and quantification of cellular elements (cellular infiltrate, blood vessels and extracellular matrix). The results demonstrated that both inhibitors prevented bone loss induced by periodontitis ($p<0.05$), and reduced the number of osteoclasts by almost 30%. Significant reduction in cellular infiltrate, as well as an increase in collagen matrix and blood vessels were found in animals treated with inhibitors ($p<0.05$). Corroborating the stereometric analysis data, JAK inhibitors also reduced the amount of CD45+ cells (leukocyte marker) in the gingival tissue. Significant reduction in the expression of inflammatory markers (at gene and protein levels) was also found in the gingival tissue of animals treated with pharmacological inhibitors. Additionally, in vitro experiments investigated the effect of JAK inhibitors on the differentiation of osteoclasts (RAW 264.7) induced by RANKL, production of reactive oxygen species (ROS), as well as on the formation of mineralization nodules and proliferation of pre- osteoblastic (MC3T3-E1). Using fluorescence microscopy, we observed that both inhibitors demonstrated a significant reduction in osteoclast formation. The data also indicated that JAK inhibition inhibited ROS production after LPS stimulation. Regarding osteogenesis, it was observed that JAK inhibition stimulated cell proliferation and the formation of mineralization nodules. The results demonstrate that the inhibition of JAK isoforms had a significant impact on reducing inflammatory changes in animals with periodontitis, indicating the leading role of the pathway in the pathogenesis and progression of the disease, in addition to the relevant role on bone metabolism cells.

Keywords: Periodontitis. Janus Kinase inhibitors. Signal transduction. Immunomodulation.

ABREVIATURAS

Acp6 - Fosfatase Ácida 5 Resistente ao Tartarato

CEUA – Comitê de Ética em Uso Animal

Col1A1 – Colágeno 1 – alpha 1

CONCEA – Conselho Nacional de Controle da Experimentação Animal

Ctsk – Catepsina K

DMEM – Meio de Eagle Modificado por Dulbecco

FBS – Soro Fetal Bovino

FDA – Food Drug Administration

Gapdh – Gliceraldeído- A 3-fosfato desidrogenase

H&E – Hematoxilina e Eosina

IFN – Interferon

IL – Interleucina

JAK – Janus Kinase

JAKi – Inibidor de JAK

LPS – Lipopolissacarídeo

MMP – Metaloproteinase de Matriz

Nfatc1 – Fator Nuclear de Células T Ativadas-Citoplasmática 1

Oscar - Receptor Semelhante à Imunoglobulina Associado a Osteoclastos

P.gingivalis – *Porphyromonas gingivalis*

RANKL - Ativador do Receptor do Ligante do Fator Nuclear *kappa*-B

ROS – Espécies Reativas de Oxigênio

SOCS – Supressores da Sinalização de Citocinas

STAT – Transdutor de Sinal e Ativador de Transcrição

TNF – Fator de Necrose Tumoral

αMEM – Meio Essencial Mínimo *Alpha*

µCT – Microtomografia Computadorizada

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal é uma condição inflamatória crônica, resultante de uma resposta imune inadequada a um biofilme microbiano disbiótico[1]. A reação do hospedeiro é iniciada e modificada por múltiplos genes, em combinação com fatores relacionados ao estilo de vida e ambientais. Albergados neste biofilme, as bactérias liberam fatores de virulência, lipopolissacarídeos (LPS) e antígenos, ativando citocinas, quimiocinas e fatores de transcrição, que desempenham um papel crítico na destruição dos tecidos periodontais e na reabsorção óssea alveolar[2,3].

A modulação da resposta imunológica pode restabelecer o equilíbrio entre substâncias que promovem e inibem a inflamação, interrompendo o avanço da doença e criando um ambiente propício para resolução da inflamação[4,5]. Além disso, o controle da inflamação através da regulação da resposta imunológica, limita o fornecimento de nutrientes para a microbiota e favorece a reversão da disbiose, estimulando o restabelecimento de uma comunidade microbiana compatível com a saúde periodontal[4,5].

Vários estudos clínicos e pré-clínicos têm examinado a eficácia de agentes moduladores da resposta imunológica no tratamento adjunto da periodontite[6]. O uso de substâncias como bifosfonatos[7], suplementos de ômega-3[8], polifenóis como o curcumin[9,10] e o resveratrol[11], bem como doses subantimicrobianas de doxiciclina e anti-inflamatórios não esteroidais[12], têm mostrado algum benefício quando usados em conjunto com a raspagem e alisamento radicular, diminuindo a progressão da doença e/ou promovendo a resolução da inflamação.

De fato, a atividade biológica das citocinas afeta diretamente e indiretamente a extensão e a gravidade da periodontite[13]. Uma maior expressão de mediadores inflamatórios são encontrados em sítios afetados pela periodontite em comparação aos sítios saudáveis, e o desequilíbrio entre a produção de marcadores pró e anti-inflamatórios é um dos principais fatores envolvidos na destruição dos tecidos periodontais[4,5].

1.1 Vias Intracelulares como Alvos Terapêuticos

Considerando o papel biológico das citocinas na origem e progressão de doenças inflamatórias[2,14,15], terapias imunomoduladoras baseadas na inibição

específica de determinados mediadores (como RANKL, IL-6, TNF e MMPs), têm sido bem-sucedidas no tratamento clínico de várias doenças, como artrite reumatoide, câncer e osteoporose[16,17], bem como em modelos pré-clínicos de periodontite. Corroborando estes resultados, demonstramos que o silenciamento de MMP-13 nos tecidos periodontais através da aplicação local de um shRNA adenoviral, reduziu significativamente a reabsorção óssea induzida por lipopolissacarídeo (LPS) bacteriano em ratos, e inibiu os níveis de mRNA e proteína de TNF-α e IL-6[18]. Embora efeitos indesejáveis não tenham sido observados nos animais durante o período experimental, uma vez que o vetor adenoviral permite silenciamento rápido e transitório da citocina alvo[18], a maioria das drogas utilizadas para inibição de citocinas estão associadas ao aparecimento de efeitos secundários, como hemorragia, problemas gastrointestinais, renais ou hepáticos[19].

Uma alternativa para modular a produção exagerada de marcadores inflamatórios é a regulação das vias de sinalização intracelular, que são cruciais para a sua expressão[19]. Uma das vantagens dessa abordagem é que a expressão de muitos mediadores inflamatórios requer a ativação de um número limitado de vias de sinalização, e a regulação de uma única via pode afetar toda uma rede de citocinas. Além disso, como característica chave da sinalização de citocinas, a ativação das vias é um processo geralmente rápido e temporário, o que permite que a regulação de uma via tenha um impacto significativo sobre a expressão de citocinas, sem causar efeitos adversos significativos em processos celulares fisiológicos[19].

Uma das vias responsáveis pela expressão da maioria das citocinas que participam da doença periodontal é a via Janus Kinase/Transdutor de Sinal e Ativador de Transcrição (JAK/STAT)[20]. JAK medeia a transdução de sinal de aproximadamente 60 diferentes citocinas, hormônios e fatores de crescimento, incluindo fatores reguladores do sistema imune e hematopoiéticos, e desta forma, participa de uma série de eventos celulares, como proliferação, ativação e sobrevivência celular[20,21].

A família JAK inclui quatro tirosinas quinases (JAK1, JAK2, JAK3 e TYK2), altamente conservadas e não redundantes. As isoformas podem se associar de forma seletiva à porção citoplasmática de receptores transmembrana, e quando ativadas fosforilam múltiplos substratos, incluindo os transdutores de sinal e ativadores de transcrição (STATs). Estes por sua vez, dimerizam e se translocam do citoplasma para

o núcleo, agindo como fatores de transcrição e regulando a expressão de vários genes que participam da resposta inflamatória[21].

Mamíferos apresentam sete tipos de STATs (STAT1-4, 5A, 5B e 6), e embora sejam ativados por subconjuntos sobrepostos de citocinas, diferentes STATs podem ter papéis biológicos não redundantes. Desta forma, os efeitos da ativação da via JAK / STAT são influenciados de forma seletiva pela isoforma de JAK envolvida, e os STATs que são finalmente ativados[22].

1.2 Regulação Endógena da Via JAK/STAT

A regulação negativa da via JAK/STAT é controlada por uma família de proteínas crítica na transdução de sinais da inflamação, as proteínas supressoras de sinalização de citocina (SOCS). A família de proteínas SOCS é constituída por 8 membros que podem ser ativados por citocinas pró e anti-inflamatórias, que interferem na sinalização JAK/STAT através de feedback negativo[23]. Essa regulação é importante, uma vez que o estímulo excessivo por citocinas induzidas pela sinalização JAK/STAT, tem sido associado a doenças inflamatórias, como artrite reumatoide, esclerose múltipla e doenças inflamatórias intestinais[20,21].

Citocinas envolvidas na patogênese da artrite reumatoide, e.g. IL-6 e TNF-α, ligam-se a receptores específicos que são capazes de ativar a sinalização JAK/STAT. A ativação da via pode induzir resistência à apoptose de fibroblastos sinoviais, aumentando a quantidade destas células[24], além de regular a expressão de citocinas e quimiocinas capazes de provocar um dano tecidual direto[25]. Essas mesmas observações têm sido propostas para a periodontite.

1.3 Inibição Farmacológica de JAK

Os inibidores farmacológicos de JAK são pequenas moléculas capazes de bloquear a sinalização de citocinas, interferindo diretamente com a atividade enzimática da proteína. São passíveis de administração via oral, e por possuírem meia-vida plasmática curta, ausência de imunogenicidade e farmacocinética linear, tem atraído atenção frente ao uso de anticorpos monoclonais no tratamento de doenças inflamatórias[20], além de ser uma alternativa ao uso de glicocorticoides e outros imunomoduladores (como metrotexato), que estão relacionados a taxas de reincidência em torno de 30%[20,26].

Estudo em ratos investigando o efeito de JAK sobre a reabsorção óssea em modelos de artrite reumatoide, observou que a inibição farmacológica (via oral) da via, reduziu o edema, a inflamação e a reabsorção óssea nas patas dos animais, sem causar efeitos secundários. Redução nos níveis de RANKL e IL-6 (transcritos e proteicos) também foram observados[25]. O inibidor não afetou a atividade dos osteoclastos, mas reduziu os níveis de RANKL produzidos por linfócitos T de maneira dose-dependente[25]. Em outro estudo com o mesmo desenho experimental[27], redução na severidade da inflamação, escores físicos da doença, níveis da proteína C-reativa, IL-1β e células T CD4 esplênicas também foram relacionados aos efeitos da inibição de JAK.

Considerando a importância da ativação de JAK para a progressão da artrite, recentemente o uso de inibidores de diferentes isoformas foi aprovado pela Food Drug Administration (FDA) para o tratamento de pacientes refratários a outras drogas. Dados de mais de 4 mil pacientes utilizando inibidor de JAK1-3 para tratamento da artrite reumatoide mostrou que o grupo tratado apresentou melhora clinicamente significativa nos sinais e sintomas da doença, ao longo de 8 anos de acompanhamento[28].

1.4 Evidência do Papel da Via JAK/STAT na Doença Periodontal

Na doença periodontal, informações sobre a expressão de JAK, bem como seu papel na progressão da doença, são escassos. Um único estudo avaliando o efeito da inibição de JAK3 em modelo experimental de periodontite, descreveu aumento na densidade do infiltrado inflamatório e na perda óssea alveolar em camundongos que receberam a inoculação de *Porphyromonas gingivalis* (*P. gingivalis*) e administração oral do inibidor[29], indicando um papel anti-inflamatório para JAK3 na progressão da doença. De maneira semelhante, células imunes estimuladas com *P. gingivalis* e tratadas com o inibidor de JAK aumentaram a produção de citocinas inflamatórias em relação ao controle, *in vitro*[29].

Diferentemente do estudo em murinos, um estudo clínico comparando a condição periodontal de pacientes com artrite reumatoide no baseline e 24 semanas após tratamento via oral com inibidor de JAK1-3, observou melhora consistente na severidade da artrite reumatoide e no status periodontal ($p<0.05$), indicando que a

inibição de JAK é de fato eficiente para tratar pacientes com artrite e potencialmente capaz de modular o processo inflamatório nos tecidos periodontais[30].

Os resultados conflitantes acerca do papel de JAK na patogênese da doença periodontal, associados ao potencial dos inibidores desta via no tratamento de diversas enfermidades inflamatórias, indicam a necessidade de realização de mais estudos que possam fornecer conhecimentos que embasem novas abordagens terapêuticas no controle adjunto da periodontite em pacientes de risco e/ou refratários ao tratamento periodontal básico.

2 PROPOSIÇÃO

O objetivo deste estudo é avaliar o impacto da inibição de JAK in vivo e in vitro durante a progressão da periodontite.

2.1 Objetivo Geral

Investigar o papel de JAK na progressão da periodontite em ratos, e seu impacto sobre a diferenciação e atividade de células relevantes do microambiente periodontal, através da inibição farmacológica de suas isoformas (JAK1-3, JAK3).

2.2 Objetivos Específicos

a) Realizar uma revisão de literatura abordando o histórico de inibidores de JAK previamente empregados no tratamento de doenças osteolíticas, com objetivo de adquirir um conhecimento mais detalhado sobre as dosagens aplicadas e os resultados obtidos tanto em estudos *in vivo* quanto *in vitro*, além de investigar os desfechos de ensaios clínicos relevantes (**Publicação 1**).

b) Avaliar *in vivo* o efeito da inibição de JAK sobre a progressão da periodontite. Ratos com doença periodontal induzida por ligadura, receberam sistemicamente (via oral) os inibidores de JAK1-3 e JAK3 para avaliação dos seguintes desfechos: extensão da perda óssea por microtomografia computadorizada (µCT), imunolocalização do infiltrado inflamatório (CD3 e CD45), bem como identificação e quantificação de osteoclastos presentes nos tecidos gengivais (morfometria); detecção da expressão dos genes inflamatórios (Il-6, Rankl e Tnf-α) por RT-qPCR e dos níveis de TNF-α através do teste ELISA (**Publicação 2**).

c) Investigar *in vitro* os efeitos da inibição de JAK sobre a osteoclastogênese (por meio de experimentos de diferenciação dos osteoclastos por microscopia de fluorescência), bem como sobre a osteoblastogênese (através de experimentos de proliferação celular e formação de nódulos de mineralização celular em pré-osteoblastos); avaliar o efeito da inibição de JAK sobre expressão gênica de marcadores de reabsorção óssea (Nfatc1, Oscar, Acp5, Mmp-9 e Ctsk); em células

pré-osteoclásticas estimuladas com RANKL,; verificar o efeito dos inibidores sobre a produção de espécies reativas de oxigênio (ROS) em macrófagos; bem como sobre a migração, proliferação e expressão gênica (Il-6, Il-10, Il-1β, Tnf-alpha, Il-17, Cola1a) em fibroblastos gengivais (**Publicação 3**).

d) Comparar a eficácia dos inibidores de JAK1-3 e JAK3 nos desfechos avaliados.

3 PUBLICAÇÕES

A tese em questão deu origem aos artigos abaixo.

3.1 Publicação 1*

Review

JAK/STAT as a Potential Therapeutic Target for Osteolytic Diseases

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Abstract: Several cytokines with major biological functions in inflammatory diseases exert their functions through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signal transduction pathway. JAKs phosphorylate the cytoplasmic domain of the receptor inducing the activation of its substrates, mainly the proteins known as STATs. STATs bind to these phosphorylated tyrosine residues and translocate from the cytoplasm to the nucleus regulating further the transcription of several genes that regulate the inflammatory response. The JAK/STAT signaling pathway plays a critical role in the pathogenesis of inflammatory diseases. There is

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also increasing evidence indicating that the persistent activation of the JAK/STAT signaling pathway is related to several inflammatory bone (osteolytic) diseases. However, the specific mechanism remains to be clarified. JAK/STAT signaling pathway inhibitors have gained major scientific interest to explore their potential in the prevention of the destruction of mineralized tissues in osteolytic diseases. Here, our review highlights the importance of the JAK/STAT signaling pathway in inflammation-induced bone resorption and presents the results of clinical studies and experimental models of JAK inhibitors in osteolytic diseases.

Keywords: JAK/STAT inhibitor; Osteoclasts; Osteoblasts; Cytokines; JAK/STAT signaling pathway; Osteolysis; Osteolytic Disease.

1. Introduction

Bone architecture is one of the most important systems in the human body, supporting tissues and protecting vital organs [1]. In addition, it is in the bone tissue that a large part of the reserves of calcium and phosphate is found, minerals of extreme importance to maintaining systemic health [1].

Bone constantly undergoes a process of resorption of older bone and formation of a new one [1]. This is characterized as bone remodeling, resulting from a balanced interplay between two important cells, osteoclasts and osteoblasts [2]. The functions of these cells are at opposite ends; while osteoblasts are responsible for forming bone tissue, osteoclasts resorb it [2]. Usually, these processes are well orchestrated so there is no discrepancy in bone mass maintaining homeostasis [2]. However, an increase or decrease in the number and/or activity of these cells is related to the development of diseases, such as inflammatory arthritis, myelofibrosis, periodontitis, osteoporosis, osteopetrosis, and bacterial-induced osteolysis, among others [1,2].

Osteoblast and osteoclast activities are regulated by numerous cytokines expressed in the bone microenvironment [3]. At the cellular level, the information transmitted by these cytokines triggers a cascade of signaling pathways that process information [3]. Among them, the Janus tyrosine kinase (JAK) and signal transducers and activators of transcription-mediated signaling (STAT) are responsible for the signal transduction

of more than fifty cytokines, growth factors, and hormones with pivotal roles in bone homeostasis [3].

Therefore, this article reviews the role of the JAK/STAT signaling pathway during bone remodelling affected by inflamation and how its inhibition can be used as a therapeutic target in osteolytic disorders.

2. JAK/STAT cell signaling pathway

The JAK-STAT pathway was originally described as primarily activated in response to interferon (IFN)-gamma and members of the interleukin-6 (IL-6) family [3]. It is now well understood that it serves as a mediator for numerous cytokines, hormones, and growth factors, suggesting that it plays a role in bone development, metabolism, and healing [4].

2.1 Family members

The JAK family includes four tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) that are selectively associated with different chains of cytokine receptors for the phosphorylation of multiple protein-derived residues [5]. The STATs, in turn, bind to these phosphorylated tyrosine residues, dimerize, and translocate from the cytoplasm to the nucleus, where it binds to DNA and can then activate or block the signal transcription [6]. There are seven types of STATs in mammals, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, and although they are activated by overlapping subsets of cytokines, different STATs may have non-redundant biological roles [6].

2.2 JAK/STAT signaling pathway regulatory mechanisms

The cell activation through the JAK/STAT signaling pathway is very fast and effective after cytokines' binding to their respective receptors [7]. JAKs become active after the multimerization of the receptor mediated by ligands; in this way, there is a phosphorylation of the main substrate, STAT [7] (figure 1).

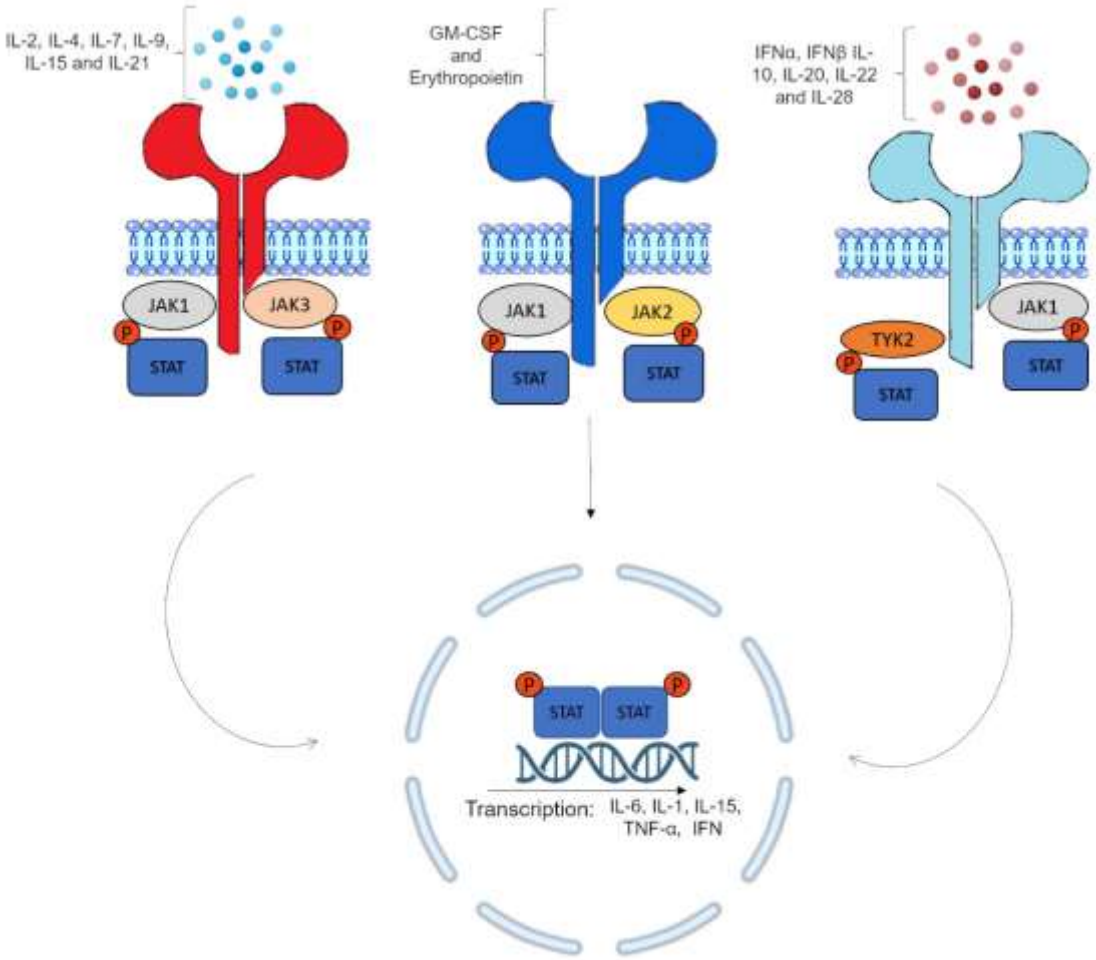

Figure 1. Potential mechanisms for activation of the JAK-STAT signaling pathway. Once the cytokines bind to their receptors with intracellular signaling via Janus kinase, it triggers an intracellular signal transducer that cause the autophosphorylation and phosphorylation of the cytoplasmic domain of cytokine receptors associates, with JAKs (JAK1, JAK 2, JAK 3 and TYK2), and also triggers the phosphorylation of the STAT proteins. JAK-mediated STAT phosphorylation results in the phosphorylation and dimerization of STAT protein, nuclear translocation, and induction of transcription of inflamatory cytokines as Interleukins (IL) -1, IL-6, IL-15, Tumor necrosis factor alpha (TNF-α), and Interferons (IFN).

A family of critical proteins in the transduction of signals and in modulation of the JAK/STAT pathway are the suppressors of cytokine signaling (SOCS) proteins [8]. The SOCS family consists of 8 proteins activated by several pro- and anti-inflammatory cytokines, and it interferes with JAK/STAT signaling through negative feedback [8]. Excessive JAK/STAT stimulation by cytokines such as IL-1, IL-6, IL-15, TNF-α, and IFN

has been associated with rheumatoid arthritis (RA), multiple sclerosis, inflammatory bowel disease, and periodontal disease, to name a few [8].
Hence, JAK/STAT signaling is crucial in initiating the innate immune response and orchestrating the mechanisms responsible for the adaptive immunity [9]. Therefore, inhibiting its activation restricts undesirable responses of inflammatory processes of various pathological conditions, many of which are of osteolytic origin [5, 9, 10].

3. JAK/STAT in bone metabolism

Several studies have shown that the JAK/STAT signaling pathway is closely related to diseases of autoimmune origin, probably by the intertwining of the immune and bone systems [11-14]. Although each bone cell has its specific function, osteoblasts directly influence the differentiation of osteoclasts through the production of several molecules, *e.g.*, the receptor activator of the nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) [11]. Physiological bone remodeling occurs through tight regulation of the RANK/RANKL/OPG triad [12]. RANKL is pivotal during bone resorption as it binds to RANK on the osteoclast precursors' surface and induces differentiation into mature osteoclasts [13]. However, this interaction is negatively regulated in the presence of OPG, as this protein has an affinity with RANKL, serving as a decoy impeding RANKL binding to RANK [12-14].

Some studies have shown that the JAK2/STAT5B pathway plays an important role in growth hormone signaling, which is essential for the differentiation of osteoblasts - cells responsible for bone formation [15-17]. This pathway is also fundamental in the formation of osteoblasts, as it acts on important transcription factors for this process, such as Runt-related transcription factor 2 (RUNX-2), Bone morphogenetic protein-7 (BMP-7) and T-Box transcription factor 3 (TBX-3). These transcription factors are important proteins in the formation of osteoblasts because they regulate the expression of genes involved in bone formation, stimulating the production of proteins necessary for bone mineralization and the formation of an adequate extracellular matrix. Furthermore, the role of the JAK2/STAT5B pathway in its regulation may lead to an increase in osteoblast formation and, therefore, in bone formation [12, 18-20].

Further research has demonstrated the crucial role of JAK activation in osteoclasts and, consequently, in bone resorption [21, 22]. In a study, a selective JAK2 inhibitor, AG490, was utilized both in vivo and in vitro, resulting in positive outcomes for

osteogenesis and bone healing following induced bone defects [21]. On the other hand, another study investigated the effects of JAK2 inhibition with ixequizumab in patients with axial spondyloarthritis, an inflammatory condition that can lead to bone resorption. The patients received a dose of 80mg every two weeks and showed positive results, including reduced disease progression [22].

Genetically JAK1 deficient mice present less bone growth and lower body mass, fail to nurse despite a normal nurturing behavior of the mothers and die perinatally [23]. Corroborating this result, in another study, JAK1 knockout mice died during the perinatal period, showing a significantly smaller skeletal structure when compared to counterparts without the deficiency [23].

In addition to the importance of bone cells, it is worth highlighting the role of cytokines that are also involved during the inflammatory process and their relationship with the JAK/STAT signaling pathway [24]. Studies report that with the activation of STAT3 and the receptor subunit gp130, IL-6 can inhibit the differentiation of osteoclasts by RANKL [24]; however, it stimulates the production of IL-1 [25], and RANKL in osteoblasts inducing greater bone resorption [26,27].

Through STAT6 phosphorylation, there is an inhibition of osteoclast activity by IL-4, and conversely, through phosphorylation of STAT5, IL-7 induces the formation of osteoclasts [28]. IL-3, on the other hand, stimulates the process of osteoclastogenesis probably through the JAK2/STAT5 pathway [28-30] and IL-17A through the JAK2/STAT3 pathway [31-33].

Finally, it is important to emphasize that not all JAKs and STATs are equally potent regulators of bone biology, which means that tuning the level of bone remodeling may be possible by acting on specific JAKs and/or STATs [1]. Further studies shall explore their modulation or inhibition capacities as a therapeutic target in inflammatory disorders of osteolytic origin.

4. Inhibition of the JAK/STAT pathway as a therapeutic target

Drugs that inhibit the JAK/STAT pathway to reduce signs and symptoms from inflammatory diseases have gained great interest. Some are already marketed and increasingly subjected to research on their effects at different target sites [34]. The

delay in the ample use and commercialization of JAK inhibitors are widely related to purchase costs, which differ broadly among countries and even internally due to fluctuations caused by seasonal demands [35]. For example, the price is fixed in France, but it is based on free demand in the United States, JAK inhibitors are generally more expensive than conventional synthetic disease-modifying antirheumatic drugs (DMARDs - methotrexate, sulfasalazine, leflunomide and hydroxychloroquine) but are comparable in price to biologic DMARDs (infliximab, adalimumab, etanercept, abatacept and tocilizumab) [35].

The JAK inhibitors that are currently approved by the Food and Drugs (FDA) and the European Medicines Agency (EMA) are Ruxolitinib (Jakavi®), which aims to inhibit JAK1 and JAK2 in patients with myelofibrosis and polycythemia vera, Tofacitinib (Xeljanz, Jakvinus, CP-690550) which inhibits JAK1 and JAK3 in rheumatoid arthritis and psoriatic arthritis, and finally, Baricitinib, targeting JAK1 and JAK2, for the treatment of rheumatoid arthritis [35]. Other JAK inhibitors are currently the subject of numerous studies, such as Pefacitinib, Filgotinib, Upacitinib, Itacitinib, Momelitinib, Gandotinib, Lestaurtinib, Decernotinib, Filgotinib, and Pacritinib, and showed promising results in Phase III clinical trials in rheumatoid arthritis, atopic dermatitis disease, Crohn's disease or myelofibrosis [35-38].

There are adverse effects reported for JAK inhibitors, mainly associated with Tofacitinib, *e.g.*, malignancy, non-viral opportunistic infections, gastrointestinal perforation, and herpes zoster [36]. However, a greater use of these inhibitors in a longer exposure time will bring more evidence about the risks associated with these compounds [36, 39-42].

We will address the results of inhibitors of the JAK/STAT signaling pathway reported in the literature in bone diseases that affect a large part of the world population.

5. JAK/STAT inhibitors in pathological bone diseases

5.1 Rheumatoid arthritis

One of the most prevalent bone diseases is rheumatoid arthritis, a chronic systemic inflammatory condition characterized by joint destruction, progressively affecting the synovial lining of the joints, causing pain, deformity, and loss of function in the affected

limbs [43]. Conventional (such as methotrexate and sulfasalazine) and biological (TNF and IL-6 inhibitors) DMARDs have been the mainstay for treating patients with rheumatoid arthritis [44]. However, due to the lack of efficacy and side effects, new drugs were explored to find more viable alternative treatments for the disease [43,44].

JAK3 inhibitor was approved by the FDA as a therapeutic strategy for rheumatoid arthritis as an alternative for patients who do not respond well to other drugs [45]. The inhibitor tofacitinib has been shown to decrease the production of IL-17 and IFN-γ and the proliferation of CD4+ T cells in cells derived from patients with arthritis [46] (table 1). A systematic review and meta-analysis evaluating the results of more than 4,000 patients using JAK3 inhibitor for the treatment of rheumatoid arthritis concluded that the treated group showed significant improvement in signs and symptoms compared to other groups [47]. Moreover, the inhibitor presented a safe pharmacological profile and prolonged efficacy for up to 8 years of follow-up [47]. Side effects related to immunosuppression have been reported by some patients and are related to the development of opportunistic infections, such as herpes zoster and respiratory infections; however, the risk for the development of these complications is the same presented by the administration of other DMARDs [48]. Preliminary studies in rats also suggest an increase in bone mass, compatible with a reduction in the RANKL/OPG ratio in serum, as well as an increase in osteoblast function after the administration of tofacitinib and baricitinib [49] (table 2).

In a randomized, double-blind, placebo-controlled, parallel-group clinical trial, final scores of patients with rheumatoid arthritis improved, as well as greater inhibition of radiographic progression of bone loss was seen with oral ingestion of tofacitinib 10 mg and 5 mg, twice a day, daily, for 3 months [45, 50]. Consistently, another study in patients with inadequate response or intolerance to conventional synthetic or biologic DMARDs demonstrated a significant improvement in rheumatoid arthritis signs and symptoms after 24 weeks with the use of baricitinib prescribed according to local recommendation for biologic and targeted synthetic therapyaligned with European League Against Rheumatism (EULAR) guide-lines (<7.5 mg/day) [51].

By evaluating the use of the JAK inhibition Tofacitinib in a rat adjuvant-induced arthritis (AIA) model, LaBranche et al. obtained satisfactory results, such as reduction of edema, inflammation, bone resorption, and plasmatic RANKL and IL-6 levels [50]. *In*

vitro results corroborated the decrease in RANKL levels by T lymphocytes after treatment with JAK inhibitor [52].

In rats with adjuvant-induced arthritis, tofacitinib markedly reduced the clinical status of treated rats compared to the control group [53]. The main findings were a reduction in joint inflammation and a decrease in serum C-reactive protein levels which was reflected in a significant reduction in mean paw diameter and an increase in body weight. Furthermore, tofacitinib significantly reduced the frequency of Clusters of Differentiation 4 (CD4+), IFN-γ+, T cells and the levels of IL-1β mRNA expression in the spleen of treated rats [53].

Some recent studies provide valuable information about the efficacy and safety of different treatment options for rheumatoid arthritis and their impact on patient reported outcomes (PROs) and disease progression [54-58]. Research has focused on identifying distinct trajectories of disease activity in methotrexate-naïve patients with rheumatoid arthritis receiving tofacitinib for 24 months [54]. The results revealed that treatment with tofacitinib (5mg twice daily) led to significant improvements in disease activity, with the majority of patients experiencing sustained low activity or disease remission. However, a small proportion of patients exhibited trajectories of high disease activity, suggesting the need for individualized treatment approaches [54]. In contrast, two studies examined the impact of initial therapy with upadacitinib (15mg/day) or adalimumab on achieving treatment goals in patients with RA. Both studies demonstrated that treatment with upadacitinib led to significantly higher rates of achieving clinical remission or low disease activity compared with adalimumab [55, 57]. Still based on the results of another study that focused on PROs in Asian patients with RA treated with peficitinib (100 and 150mg/day), demonstrating significant improvements in pain, physical function and other PROs compared to placebo [56]. However, a post hoc analysis of a Japanese phase 3 study of peficitinib (100mg/day) and methotrexate showed positive results in reducing radiographic progression, even though some patients did not respond to treatment, indicating the need for further investigations into optimal treatment strategies [58].

Overall, these studies highlight the importance of individualized treatment approaches for RA and the need to consider clinical and PRO when assessing treatment efficacy. Furthermore, the results suggest that newer treatments such as upadacitinib and

tofacitinib may provide superior efficacy compared to traditional treatments such as methotrexate and adalimumab.

A better understanding of JAK/STAT pathway activation for the occurrence and development of rheumatoid arthritis will allow the deepening of strategies and therapeutic targets and the development of JAK inhibitor antirheumatic drugs.

5.2 Myelofibrosis

Myelofibrosis is characterized by an overproduction of myeloid stem cells, bone marrow fibrosis, cytopenia, and extramedullary hematopoiesis, which exacerbates the release of inflammatory cytokines [59]. The only curative treatment for myelofibrosis is allogeneic hematopoietic stem cell transplantation. Still, because of life-threatening risks, especially for elderly patients and those with comorbidities, new therapeutic targets have been studied as alternative treatments for the disease [59].

A promising example is AZD1480 which blocked at low micromolar concentrations cell proliferation and induced apoptosis of myeloma cell lines via concomitant inhibition of the phosphorylation of signaling proteins JAK2, STAT3, and MAPK [60]. In clinical trials, an improvement in the symptoms of patients using ruxolitinib was observed due to the reduction in the degree of bone marrow fibrosis and normalization of bone lesions at concentrations of 20mg for 16 weeks in one study and 15 mg for 12 months in another, both used 2 times a day, daily [61 62]. In another study, the continued therapy with ruxolitinib (initially prescribed at a dose of 15 or 20 mg twice a day depending on the baseline platelet count) was associated with marked and lasting reductions in splenomegaly and disease-related symptoms, improved quality of life, and modest toxic effects [63].

A problem associated with myelofibrosis is the appearance of infectious diseases [48]. A reduction in the cytokine-induced activity and function of natural killer cells of patients with myelofibrosis was observed [48], suggesting a potential preventive role of ruxolitinib in secondary infections.

Some recent studies have investigated different treatments for myelofibrosis, each targeting different disease pathways [64-66]. The first study compared the effectiveness of momelotinib (100 and 200mg/day) and danazol in relieving anemia and other symptoms in patients with myelofibrosis. The study found that both drugs improved symptoms, but momelotinib was associated with fewer adverse events and

better splenic response rates than danazol [64]. On the other hand, the study of fedratinib (300, 400 and 500mg/day) found that the drug was effective in treating myelofibrosis in patients with low platelet counts, a common complication of the disease. The drug was generally well tolerated, with few serious adverse events reported [65]. Finally, a study on jaktinib (100 and 200mg/day) evaluated the safety and efficacy of the drug in Janus kinase inhibitor-naïve patients with myelofibrosis. The drug was well tolerated and effective in reducing spleen size and improving symptoms, with no unexpected safety concerns [66].

Overall, these studies highlight the importance of targeted therapies in the treatment of myelofibrosis and suggest that different drugs may be appropriate for different patient populations, depending on individual disease characteristics.

Further research using the JAK/STAT signaling pathway inhibitors should be carried out to define a protocol for clinical application in myelofibrosis.

Table 1. Summarization of the findings from JAK-STAT inhibitors in *in vivo* and *in vitro* studies

Drugs*	Disease	Outcomes
Inhibitor AZD1480 [60]	Myelofibrosis	*in vitro:* - Blocking of cell proliferation and induction of apoptosis of myeloma cell lines; - Cell death of KMS-11 cells grown in the presence of HS-5 bone marrow-derived stromal cells - Iinhibition of tumor growth in a KMS-11 xenograft mouse model, accompanied with inhibition of phospho-FGFR3, phospho-JAK2, phospho-STAT3 and Cyclin D2 levels;
Tofacitinib [46]	Rheumatoid Arthritis	*in vitro:* - Inhibition of the production of IL-17 and IFN in a dose-dependent manner; - Effect in proliferation and transcription;
Baricitinib [52]	Rheumatoid Arthritis	*in vivo:* - Reduction of edema; - Reduction of inflammation and bone resorption;

		- Reduction of RANKL and IL-6 levels; *in vitro:* - Did not affect osteoclast function and activity; - Decreased RANKL levels produced by T lymphocytes in a dose-dependent manner
Tofacitinib [67]	Rheumatoid Arthritis	*in vivo:* - Reduction of the clinical status of treated rats in comparison to control group. - Reduction of joints inflammation and down-regulated serum CRP levels reflected the clinical manifestations of the treated rats. - Tofacitinib down-regulated significantly the frequency of CD4+IFN-γ+ T cells and reduced IL-1β mRNA expression levels in the spleen of the treated rats;
Tofacitinib [49]	Rheumatoid Arthritis	*in vivo:* - Reduction in the severity of inflammation and in the physical score of arthritis; - Reduction in C-reactive protein levels; *in vitro:* - Reduction of splenic CD4 T cell levels;
Ruxolitinib [48]	Myelofibrosis	- Reduction in NK cell numbers; - Endogenous functional defect of NK cells in MPN were further aggravated; - Reduction in cytokine-induced NK cell activation; - Reduced killing activity of primary NK cells was associated with an impaired capacity to form lytic synapses with NK target cells; - Ruxolitinib impairs NK cell function in MPN patients;
Baricitinib [49]	Rheumatoid Arthritis	*in vivo:* - Increased bone mass, consistent with reducing the ratio of receptor activator of NF-ƙB ligand/osteoprotegerin in serum; *in vitro:* - Increased osteoblast function but no direct effects on osteoclasts;

Abbreviations: IL: Interleukins; INF: Interferon; RANKL: Receptor Activator of Nuclear Factor-kappa Be ta Ligand; NF-ƙB: Factor Nuclear Kappa B: STAT: Signal Transducer and Activator of Transcription; JAK: Janus Kinase; NK cells: Natural Killers cells: MPN: Myeloproliferative Neoplasms.

Table 2. Summarization of the findings from clinical studies on JAK-STAT inhibitors

Drugs	Disease	Outcomes	Side Effects
Ruxolitinib [63]	Myelofibrosis	- Reduction of spleen volume; - Improved quality of life; - Improvement of the symptoms of the disease;	Diarrhea, peripheral edema, asthenia, dyspnea, nasopharyngitis, pyrexia, cough, nausea, arthralgia, fatigue, pain in extremity, abdominal pain, headache, back pain, pruritus.
Ruxolitinib [61]	Myelofibrosis	- Improvement in symptoms and other signs of myeloproliferation;	None reported
Ruxolitinib [62]	Myelofibrosis	- Reduction of bone marrow fibrosis grade and resolution of osteolytic lesions;	None reported
Momelotinib [64]	Myelofibrosis	- Improvement in quality of life, reduction in the need for blood transfusions, and improvement in fatigue, pain, early satiety and weight loss;	- Anemia, thrombocytopenia, nausea, diarrhoea, headache, fatigue, arthralgia;
Fedratinib [65]	Myelofibrosis	- Increased platelet count and reduction in splenomegaly;	- Diarrhoea, nausea, anemia and headache;
Jaktinib [66]	Myelofibrosis	- Improvement of fatigue, pain and gastrointestinal symptoms; - Inhibition of the activity of the JAK2 protein in the cells of patients with proliferation of myelofibrotic cells;	- Anemia, nausea and diarrhea;

Tofacitinib [45, 50]	Rheumatoid arthritis	- Improvement in: * American College of Rheumatology scale; * Erythrocyte sedimentation rate; * hsCRP levels; * Health Assessment Questionnaire-Disability Index; - greater inhibition of radiographic signs of disease progression	Report of viral infections (Herpes Zoster) and gastrointestinal disorders
Baricitinib [51]	Rheumatoid arthritis	- All clinical parameters of rheumatoid arthritis decreased significantly (DAS28-CRP, SDAI, ESR, CRP, ACPA and FR);	None reported
Tofacitinib [54]	Rheumatoid arthritis	- Reduction of joint swelling; - Reduction in C-reactive protein levels; - Improvement of reported quality of life;	None reported
Upadacitinib [55]	Rheumatoid arthritis	- Improvement in disease activity scores, pain and functional disability	None reported
Peficitinib [58]	Rheumatoid arthritis	- Significant improvements in Disease Activity Score (DAS), American Criteria Response (ACR), Patient Reported Outcomes (PROs) and Medical Reported Outcomes (MROs);	- Headache, nasopharyngitis, diarrhea, nausea and increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST), but symptoms well tolerated.
Upadacitinib [57]	Rheumatoid arthritis	- Greater improvement in joint pain, morning stiffness, fatigue and quality of life;	- Respiratory infections, nasopharyngitis and headache;
Peficitinib [58]	Rheumatoid arthritis	- Reduction of radiographic progression;	- Upper respiratory tract infections, anaemia, decreased white blood cell count, increased liver enzymes,

			headache, pneumonia and shingles;
Tofacitinib [68]	Periodontal disease	- Tofacitinib therapy reduced periodontal inflammation as indicated by the mean values of the gingival index, pocket depth, clinical attachment level, percentage of sites with bleeding on probing; - Serum levels of rheumatoid factor, matrix metalloproteinase-3, and IL-6 were decreased compared to the values at baseline;	None reported
Baracitinib [51]	Periodontal disease	- Patients with chronic periodontitis showed a significant decrease in periodontal inflammation as suggested by improvement in the number of sites with bleeding on probing and pocket depth compared to the values of baseline;	None reported

Abreviations: hsCRP: High sensitivity C-reactive protein; DAS28-CRP: Disease Activity Score including 28 joints using C-Reactive Protein; SDAI: Simplified Disease Activity Index; ESR: Erythrocyte Sedimentation Rate; CRP: C-Reactive Protein; ACPA: Anti-Citrullinated Peptide Antibody; RF: Rheumatoid Factor; DAS: Disease Activity Index Scores, ACR: American Criteria Response, PROs: Patient Reported Outcomes and Medical Reported Outcomes (MROs); ALT: alanine aminotransferase; AST: aspartate aminotransferase.

5.3 Periodontitis

Periodontitis is one of the major causes of tooth loss in adults, affecting a high number of patients worldwide creating a major public health concern that necessitates the discovery of novel therapies [69]. Periodontitis is a chronic inflammatory condition resulting from the actions of multiple causes that leads to microenvironmental changes in the tooth-supporting tissues and exacerbates by a further dysbiosis of the microbial biofilm that deepens the inflammatory response [70]. The bacteria in this biofilm release numerous virulence factors, including lipopolysaccharides and antigens, which

activate cytokines, chemokines, and transcription factors that may contribute to connective tissuedestruction and bone resorption [71]. The standard treatment comprises professional biofilm with a personalized at-home hygiene program. However, some patients with severe forms of the disease are refractory to treatment and new host modulation therapies are necessary [72-74].

During periodontitis progression and chronification, numerous signaling pathways are activated depending on the nature of the causes [75]. Although few studies have investigated the role of JAK activation in periodontitis, STAT3 and STAT5 were shown to be activated in gingival tissues of periodontally diseased rats, indicating that the JAK/STAT pathway may play a relevant role in the pathogenesis of the disease [75].

Another study evaluated the effect of the absence of suppressor cytokine signaling proteins 3 (SOCS3) in the bone tissue of mice with periodontal disease. SOCS are a family of cell signaling molecules activated by microbial and immunological stimuli [76]. They negatively regulate cytokine signaling, inhibiting the JAK/STAT signal transduction pathway [76]. When evaluating in SOCS-3 knockout mice, the study investigated the protein's influence on the progression of experimental periodontitis. The results demonstrated that SOCS-3 regulates the production of RANKL and OPG, molecules involved in the regulation of bone resorption, and inhibits the JAK/STAT signaling pathway, thereby reducing inflammation and alveolar bone loss. The administration of a SOCS-3 agonist reduced alveolar bone loss, suggesting that the protein may represent a potential therapeutic target for periodontitis in humans [77].

In contrast, JAK3 inhibition in mice with periodontal disease had increased inflammatory infiltrate and bone resorption, indicating a protective role for JAK in the pathogenesis of periodontal diseases [78] (table 3). An increase in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity and the production of pro-inflammatory cytokines was also observed in the gingival tissue of animals treated with a JAK3 inhibitor [78]. Conversely, our research group demonstrated through an experimental model of periodontitis that rats treated with oral JAK inhibitor daily for 7 days presented suppression of the inflammatory process and alveolar bone resorption during periodontal disease induction, suggesting that modulation of this signaling pathway may be a therapeutic approach for preventing periodontitis progression [79].

A clinical study comparing the periodontal status of patients with periodontitis and rheumatoid arthritis at baseline and 24 weeks after oral treatment with a JAK1-3 inhibitor indicated a reduction in periodontal inflammation [51] Similarly, an improvement in periodontal clinical parameters was observed in two case reports of patients using the JAK inhibitor Tofacitinib at a dose of 10 mg/day [68]. In vitro, JAK 1 inhibitors have been shown to inhibit alkaline phosphatase activity in periodontal ligament cells stimulated by IL-11 and IL-6 with ascorbic acid [80, 81].

Table 3. Summarization of the findings from periodontal disease studies

Drugs		Study Model	Outcomes
JAK 3 Inhibitor [79]		*in vivo*	Suppression of the inflammatory process and alveolar bone resorption duri periodontal disease induction;
JAK 1 inhibitor [80]		*in vitro*	Alkaline phosphatase activity inhibition in IL-11/ascorbic acid stimulat periodontal ligament cells;
AG490 and JAK 1 inhibitor [81]		*in vitro*	Both AG490 and JAK inhibitor I significantly diminished ascorbic acid+IL-6/s 6R-elicited alkaline phosphatase activity;
JAK1 and STAT3 inhibitors [82]		*in vitro*	No observed effect
JAK 3 inhibitor [78]		*in vivo* and *in vitro*	*in vivo:* - Enhanced infiltration of inflammatory cells, reduced expression of Wnt3a a Dvl3 in P. gingivalis-infected gingival tissues, and increased disease severity *in vitro:* - Enhancement of nuclear factor kappa-light-chain-enhancer of activated B ce activity and the production of pro-inflammatory cytokines (TNFα, IL-6 e 12P40) in P. gingivalis-stimulated innate immune cells.

Abbreviations: IL: Interleukins; TNFα: Tumor Necrosis Factor alpha.

Till now few studies have evaluated JAK/STAT signaling pathway inhibitors in periodontal disease. However, results suggest a promising role of this pathway in regulating the pathogenesis of periodontitis, and more studies are necessary to investigate further their clinical impact in patients [83]. The adjunct use of host response modulation through the inhibition of targets, such as the JAK/STAT pathway,

in conjunction with mechanical periodontal treatment may bring remarkable benefits for the treatment of periodontitis [74, 83]. Figure 2 illustrates the potential use of JAK inhibitors in periodontal diseases (figure 2).

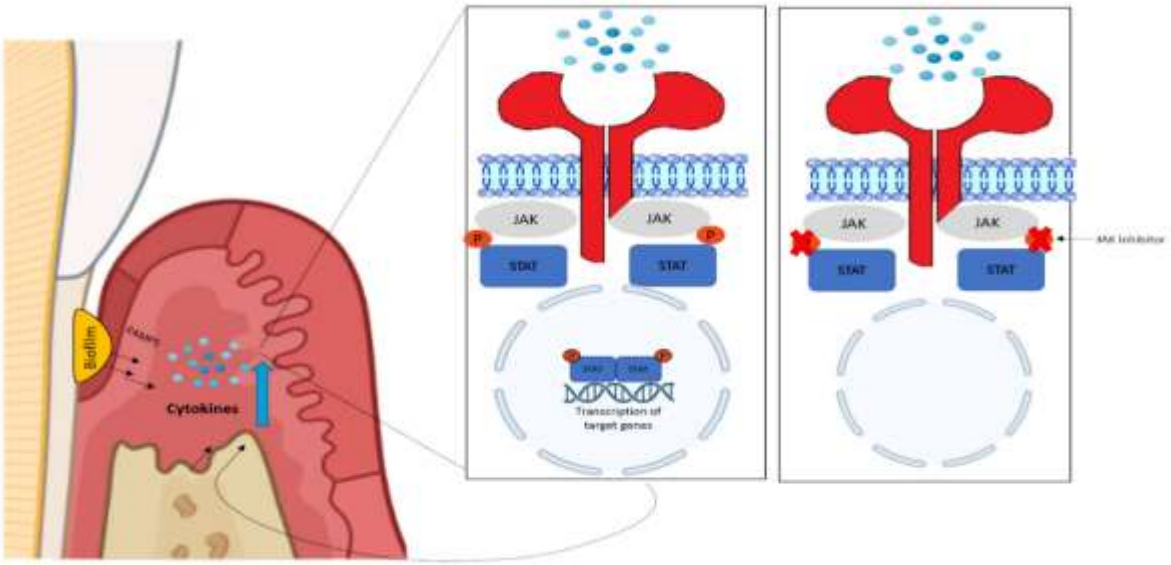

Figure 2. The recognition of microbial components (molecular patterns associated with pathogens - PAMPs) by defense cells induces an increase of inflammatory cytokines in periodontal tissues. These cytokines stimulate the activation of the JAK-STAT pathway that results in the transduction of more inflammatory cytokines amplifying the inflammatory response and tissue destruction. JAK inhibitors bind to and competitively inhibit the kinase domain of JAKs, thereby preventing JAKs from phosphorylating STATs and other substrates so that intracellular signals cannot be further transduced. Because JAKs are critical for multiple different cytokines, JAK inhibitors can block the action of a range of cytokines and contribute to the reduction of the inflammatory response and tissue destruction in the periodontium.

5.4 Osteoporosis

An important bone disease that affects a large part of the world's population is osteoporosis. [84]. Like Paget's disease, osteoporosis is of public health concern since it is a skeletal disorder characterized by compromised bone strength leading to an increased risk of fractures [84 85]. In contrast, osteopetrosis is characterized by an increase in bone mass and density due to a failure in the process of resorption of

mineralized tissues [86]. While the treatments currently approved for osteoporosis act largely by inhibiting bone resorption, such as using calcium and vitamin D, sex hormones, calcitonin, and bisphosphonates, for osteopetrosis cases to date, there is no totally effective treatment [87]. Two drugs widely used for these bone diseases are Two drugs widely used for these bone diseases are denosumab and romosozumab, both are biologic agents that have been approved for the treatment of osteoporosis [88, 89]. Denosumab is a monoclonal antibody that binds to and inhibits the activity of the receptor activator of nuclear factor-kappa B ligand (RANKL), which is a key mediator of bone resorption [88]. It has been shown to significantly reduce the risk of vertebral, non-vertebral, and hip fractures in postmenopausal women with osteoporosis. However, denosumab has been associated with an increased risk of serious infections and dermatologic adverse events [88]. Romosozumab is a monoclonal antibody that binds to and inhibits sclerostin, which is a negative regulator of bone formation [89]. It has been shown to significantly reduce the risk of vertebral fractures and increase bone mineral density in postmenopausal women with osteoporosis. However, romosozumab has been associated with an increased risk of cardiovascular events, including myocardial infarction and stroke [89], and these therapies aim to provide multidisciplinary surveillance and symptomatic treatment of complications and sometimes resort to complicated and risky bone marrow transplant surgeries [87, 90, 91].

There are still no reports using inhibitors of the JAK/STAT pathway in experimental models of osteoporosis to assess the consequences of this inhibition more specifically. However, the study conducted by Fu et al. (2020) aimed to investigate the underlying mechanism of miR-151a-3p, a small non-coding RNA molecule that plays a crucial role in regulating gene expression, in postmenopausal osteoporosis. In vitro and in vivo experiments have demonstrated that miR-151a-3p can inhibit SOCS5 expression, leading to activation of the JAK2/STAT3 signaling pathway, which results in increased osteoclastic differentiation and bone resorption [92]. Other in vivo experiments showed that overexpression of miR-151a-3p in mice resulted in decreased bone mass and increased bone resorption [92] These findings indicate that miR-151a-3p plays a significant role in postmenopausal osteoporosis and targeting it may be a potential treatment strategy for this condition. Furthermore, the study suggests that inhibition of

SOCS5 and subsequent activation of the JAK/STAT pathway is one of the mechanisms by which miR-151a-3p promotes postmenopausal osteoporosis [92].

Another successful case of using JAK/STAT pathway inhibitors was in patients with Hutchinson-Gilford progeria syndrome (HGPS), an incurable condition that affects fetuses through premature aging, causing early death [93]. Progressive joint contractures, stiffness, and osteoporosis are observed, leading to a reduced life expectancy. Ex vivo experiments with human cells demonstrated that JAK inhibition restored cellular homeostasis, delayed cellular senescence, and reduced expression levels of pro-inflammatory markers in HGPS cells [93].

Although there is limited research on the use of JAK inhibitors, these drugs show promise as a potential treatment for osteoporosis because of their ability to inhibit inflammation [94], increase osteoblast activity, and reduce osteoclast activity, which may help to prevent bone resorption [95], furthermore, JAK inhibitors can also stimulate osteogenic differentiation of mesenchymal stromal cells [96]. However, more research is needed to gain a comprehensive understanding of the mechanisms underlying the potential benefits of JAK inhibitors in osteoporosis and to assess their efficacy and safety in patients with this condition.

Persistent activation of the JAK/STAT pathway has been shown to play an important role in the pathogenesis of osteoporosis, so a deeper understanding of the mechanisms of JAK inhibitors might reveal a possible therapeutic option for a disease that affects millions of people around the world.

5. Conclusions

In recent years, a greater understanding of the signal transduction pathways involved in regulating cytokine production in immune cells has led to new discoveries in the development of therapies and treatments for various inflammatory diseases. One of these pathways, the JAK/STAT signaling pathway, has been the subject of numerous in vivo and in vitro studies due to its important role in the activity of several mediators relevant to the pathogenesis of osteolytic diseases.

This review highlights new discoveries about how the JAK/STAT pathway is intimately linked to the pathogenesis of a number of osteolytic diseases that affect a large part of the world's population. In addition, it summarizes how the inhibition of this pathway has become an attractive therapeutic target for the treatment of these disorders,

highlighting the progress achieved so far and creating momentum for further research on the subject. Recent studies have provided evidence that inhibition of the JAK/STAT pathway can effectively reduce pro-inflammatory cytokine production and prevent bone destruction in several preclinical models of osteolytic diseases.

These findings led to the development of several JAK/STAT inhibitors, some of which have already been approved for clinical use in the treatment of rheumatoid arthritis and other autoimmune diseases.

Clinical studies in patients with osteolytic diseases have shown that the use of these drugs significantly reduced the symptoms of the disease, although they can increase the risk of serious infections. In animals, JAK inhibitors have been shown to reduce the formation of scar tissue in the bone marrow, improving the symptoms of myelofibrosis. In vitro research also suggests that these drugs may help reduce inflammation in bone cells and decrease the formation of bone-destroying cells, which may hold promise for treating these diseases. However, further clinical studies in humans are still needed to confirm the effectiveness of these drugs in the treatment of periodontal disease.

Overall, these studies demonstrate the potential to target the JAK/STAT pathway as a promising therapeutic approach to use as a treatment. Therefore, it is important to conduct randomized controlled clinical trials to assess the safety and efficacy of these drugs in humans. In addition, it is important to consider the duration of treatment, proper dosage, and possible side effects of these drugs in order to determine the best clinical use of JAK inhibitors in osteolytic diseases.

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3.2 Publicação 2*

JAK inhibition prevents bone loss and reduces periodontal inflammation in an experimental rat model of periodontitis.

Running title: JAK inhibition reduces periodontal inflammation in vivo

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* O presente estudo será apresentado em formato de artigo seguindo as normas da revista *Journal of Periodontology* (Qualis A1, Fator de Impacto 4.3) para a qual será submetido.

ABSTRACT

Background: The Janus kinase and signal transducer and activator of transcription (JAK/STAT) intracellular signaling pathway regulates numerous cytokines essential in the pathogenesis of inflammatory diseases. Increasing comprehension of JAK's significance across inflammation-related illnesses has led to the development of therapeutic interventions utilizing JAK inhibitors. To evaluate the role of JAK in the progression of periodontitis through systemic administration of pharmacological inhibitors targeting JAK1-3 and JAK3 isoforms in a rat model of experimental periodontitis.

Methods: Periodontitis was induced by ligature placement around the mandibular first molars. Concurrently, animals (n=10 per group) received JAK1-3 inhibitor (JAK1-3i group), JAK3 inhibitor (JAK3i group), or distilled water (positive control group) via daily intragastric gavage for seven days. A negative control group received only distilled water via gavage without ligature placement. Following euthanasia, the mandibles were evaluated using microcomputed tomography for bone loss, stereometric analysis for inflammatory infiltrate, extracellular matrix, and blood vessels, and immunohistochemistry to quantify CD45+ and CD3+ cells. Gingival tissue was assessed for inflammatory markers by RT-qPCR (IL-6, TNF-alpha and RANKL) and ELISA (TNF-alpha).

Results: JAK1-3i and JAK3i significantly reduced bone resorption and the expression of inflammatory markers associated with periodontitis when compared to the positive control group. The inhibitors also prevented the increase in cellular infiltration and increased the amount of collagen matrix and blood vessel density ($p<0.05$) in periodontal tissues. Lower amounts of CD45+ cells were evident in the gingival tissue of animals treated with JAK1-3i.

Conclusion: Our findings support that JAK modulates alveolar bone loss and tissue inflammation in ligature-induced experimental periodontitis. Further studies are needed to explore the therapeutic impact of JAK inhibitors in periodontitis.

Keywords: Periodontitis, Janus Kinase Inhibitors, Signal Transduction, Immunomodulation, Cytokines, Bone resorption.

1. INTRODUCTION

Periodontitis is a chronic inflammatory condition resulting from an imbalanced immune response against the oral microbiome influenced by genetic, environmental and systemic causes (1). The microorganisms release virulence factors, lipopolysaccharides (LPS) and antigens, that trigger the host immune response via activation of cytokines, chemokines, and transcription factors. Although the host immune response aims to have an essentially protective role it is also responsible for most of the tissue degradation observed in periodontitis (2,3).

Currently, periodontal treatment is based on the removal of biofilm. This approach, however, does not achieve satisfactory and stable clinical results in all patients, particularly in those who develop chronic non-self-resolving inflammatory periodontal lesions. In such unsolved lesions, neutrophils resist clearance leading to a shift in macrophages towards a proinflammatory phenotype and the acquired immune response becomes activated, resulting in the accumulation of diverse lymphocytes (4). The use of complementary therapies to modulate destructive aspects of the immune response and trigger the resolution of these chronic lesions has been investigated as an alternative to achieve better long-term results (5) and provide an adjunct form of treatment.

Modulating components of the host immune response can help restore the balance between pro- and anti-inflammatory mediators, arresting periodontitis progression and promoting an environment conducive to the resolution of inflammation (1). Additionally, controlling the inflammatory process by modulating the immune response limits the supply of nutrients to the microbiota favoring the reversal of dysbiosis, and stimulates the recovery of a microbial community compatible with periodontal health (1).

Agents such as bisphosphonates, omega-3 supplements, polyphenols such as curcumin and resveratrol, as well as sub-antimicrobial doses of doxycycline and nonsteroidal anti-inflammatory drugs, have demonstrated benefits when combined with non-surgical periodontal therapy. These adjunct therapies have demonstrated the ability to reduce disease progression and promote inflammation resolution (2).

Considering the role of cytokines in the onset of inflammatory diseases (3,6,7), immunomodulatory therapies focusing on the selective inhibition of cytokines have

shown success in treating conditions such as rheumatoid arthritis, cancer, and osteoporosis. Additionally, these therapies have demonstrated efficacy in animal studies using experimental models of periodontitis (8,9). An alternative approach for controlling the overexpression of pro-inflammatory mediators involves regulating crucial intracellular signaling pathways responsible for their expression. This strategy offers the advantage that the expression of many inflammatory cytokines requires the activation of only a few signaling pathways. Regulating a single pathway can impact the expression of several cytokines. Additionally, the activation of signaling pathways is typically rapid and transient, allowing the regulation of one pathway to affect cytokine expression without causing significant adverse effects on normal cellular processes (9).

The Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins constitute the JAK-STAT pathways, essential for immune regulation (10). The JAK family includes four tyrosine kinases (JAK1, JAK2, JAK3 and TYK2), which selectively associate with the cytoplasmic portion of transmembrane receptors. Upon activation, these kinases phosphorylate multiple substrates, including STATs. Subsequently, phosphorylated STATs dimerize and translocate from the cytoplasm to the nucleus, regulating the expression of several genes with essential functions in inflammatory diseases, including periodontitis (11,12).

Considering the relevance of the JAK/STAT pathway in modulating a myriad of inflammatory cytokines, pharmacological JAK inhibitors have been developed and approved by the Food and Drug Administration (FDA) for the treatment of inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel diseases (11). Pharmacological JAK inhibitors are small molecules capable of blocking cytokine signaling, directly interfering with the enzymatic activity of the protein. They can be administered orally, and have attracted attention due to their short plasma half-life, lack of immunogenicity and linear pharmacokinetics, compared to the use of monoclonal antibodies in treating inflammatory diseases (11). They offer an alternative to conventional immunomodulators, which are associated with substantial recurrence rates (11,12).

Based on promising results in clinical and pre-clinical studies using JAK inhibitors in inflammatory osteolytic diseases, a few studies have evaluated the possible therapeutic effect of JAK inhibition on periodontal disease. Although data

indicate a relevant role for JAK in the pathogenesis of the disease, results are scarce and divergent. Therefore, the goal of this study was to investigate the therapeutic impact of JAK inhibitors on preventing the degradation of periodontal tissues during the progression of periodontitis in rats.

2. MATERIAL AND METHODS

All the experimental protocols were approved by the Ethical Committee for Animal Use (CEUA- process 27/2021) of the School of Dentistry at Araraquara – UNESP and performed in accordance with the guidelines from the Brazilian College for Animal Experimentation (COBEA). The reported data conforms to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines

Animals

Forty male rats (Rattus norvegicus albinus, Holtzman), weighing between 100-200g, (7-8 weeks) were utilized. Animals were housed in polypropylene cages (5 animals/cage) under a temperature of 21^{o}C ± 1^{o}C, humidity at 65-70%, and subjected to a 12-hour light-dark cycle. The animals received pelleted food (Labina, Purina, São Paulo, Brazil) and water *ad libitum*.

Establishment of periodontitis in rats.

The animals were subjected to the induction of periodontitis by installing ligatures bilaterally around the mandibular first molars. For ligature placement the animals were sedated with intravenous administration of 0.08 mL of ketamine [Paulinia, Sao Paulo, Brazil] and 0.04 mL of xylazine hydrochloride [Tambore, Sao Paulo, Brazil] per 100 g of body weight. The animals were positioned on an operating table for the installation of ligatures (number 24 cotton thread). The ligatures were kept in place during the seven days of the experimental period till euthanasia of the animals. All animals were euthanized by anesthetic overdose (Ketamine and Xylazine) under general inhalation anesthesia at the end of the experimental period.

Effect of JAK inhibition on periodontitis progression

To evaluate the effect of JAK inhibition during the development of periodontitis, the animals were randomly divided into four experimental groups (10 per group), as described in Supplementary Table 1: negative control (no ligature and distilled water), positive control (ligature and distilled water), JAK1-3i (ligature and JAK1-3 inhibitor) and JAK3i (ligature and JAK3 inhibitor).The sample size calculation was based on our previous data, whose primary outcome was the analysis of bone volume by bone microtomography in rats (13). Adopting a β power of 0.8 and an α power of 0.05, a minimum sample of 7 animals per group was determined. To account for potential losses during the experimental period, a sample size of 10 animals per group was used.

Two types of JAK inhibitors were administered: a commercially available selective inhibitor for JAK1-3 isoforms (CP-690-550- Tofacitinib, Cayman Chemical, MI, USA), and a JAK3 isoform-specific inhibitor (TL6-144). The JAK3 isoform-selective inhibitor used in this study was synthesized and kindly provided by Prof. Dr Nathanael Gray, Stanford University, USA. The pharmacological analysis of the JAK3 inhibitor (TL6-144) demonstrated the absence of side effects associated with oral administration and effective inhibition of JAK3 in mice (14,15). However, we evaluated for the first time the impact of JAK3 on inflammation and bone loss in periodontitis in this study.

Administration of inhibitors or distilled water started in the same day of ligature placement. The inhibitors were administered daily (6.2mg/kg), via intragastric gavage for seven days. The dose of inhibitors was determined based on previous studies using an experimental model of adjuvant-induced rheumatoid arthritis that demonstrated a significant reduction in the inflammatory infiltrate in the joints of treated animals after 4 and 7 days of oral administration, without causing any adverse effects (16,17). Mortality and physical and behavioral changes were recorded daily. Animals in the negative and positive control groups received distilled water via the same route. A vehicle group (carboxymethylcellulose 0.5%) was not included because our previous studies demonstrated the absence of any effect of the compound on outcomes similar to those evaluated in this study (13). The procedures outlined within the experimental period are represented in supplementary figure 1.

Biological samples

After euthanasia, the lower jaw of each rat was sectioned into two hemi-mandibles. Left hemi-mandibles were fixed in 4% paraformaldehyde (Sumare, Sao Paulo, Brazil) at 4°C for 18h, transferred to 70% ethanol and stored at 4°C and then used for immunohistochemical and stereometry analysis. Hemimandibles on the right side were initially immersed in 4% paraformaldehyde for 24 hours and washed in running water for 6 hours, after which time they were then immersed in 70% alcohol to be used for microcomputed tomography (μCT). Non-mineralized soft periodontal tissues around the mandibular first molars of each animal were carefully dissected, frozen in a -80° freezer and then processed by ELISA and mRNA expression analysis.

Microcomputed tomography (μCT) analysis

Right hemimandibles were further sectioned with diamond discs mounted on a mandrel at lower speed to include only the mandibular first and second molars. The remaining hemimandibles were scanned on a μCT imaging system (Skyscan 1076; Bruker, Kontich, Belgium) using an isotropic spatial resolution of 9 μm (0.01 mm), 0.5 mm aluminum filter, a voltage of 80 kV, current of 310 μA, exposure of 195 ms, frame average of 3 and rotation step of 0.4° in 180°. For reconstruction (NRecon 1.6.1.5; Bruker), the following parameters were used: ring artifact correction equal to 4, 5%-pixel defect mask, 1% straightening, 26% beam hardening correction and compressed sensor for image conversion at 0.0-0.8. The standardized region of interest (ROI) was defined in a sagittal orientation in the furcation area of the mandibular first molar, extending from the top of the furcation to the root apices (supplementary figure 2). This ROI was delimited to every ten planes with 90 slices. The analysis was performed by comparing between groups the ratio of bone volume / total volume inside the ROI (BV/TV), by a trained examiner who was blind to the experimental groups.

Histological processing

Right hemimandibles that included the lower first and second molars, were decalcified in a 10% EDTA (Diadema, Sao Paulo, Brazil) solution, pH 7.2, for 6 to 8 weeks for subsequent preparation and embedment in paraffin (Ribeirao Preto, Sao Paulo, Brazil). For the immunohistochemistry analyses, serial sections of 5 μm were obtained and mounted on silane coated glass slides. For the stereometry analyses,

semi-serial sections of 4 µm thickness were used on standard glass slides, stained with hematoxylin (St Louis, MO, USA) and eosin (Diadema, Sao Paulo, Brazil) (H&E).

Stereometry Analysis

Semi-serial sections with an interval of approximately 320 µm were used. The sections were stained with H/E and images were captured under a light microscope (Leica DM 2500, Wetzlar, Germany) with 200x magnification. For analysis, an 88-point grid was superimposed on the connective tissue corresponding area of the histological image between the lower first and second molars. This region was delimited coronally by the most apical portion of the epithelial tissue, apically by the top of the bony crest, and laterally by the most prominent region of the distal root of the first molar. A percentage analysis of tissue components including extracellular matrix, cellular infiltrate and blood vessels in the interest region was performed in relation to the total number of points counted. The analyses were performed by a trained examiner blinded to the experimental groups. The results were used to determine an average value per animal, and then these values were compared between the groups.

Immunohistochemistry

The presence of an inflammatory process represented by the number of CD45+ leukocytes and CD3+ T lymphocytes was identified and quantified between the first and second lower molars. Analyses were performed using the biotin-streptavidin-HRP-DAB method (Envision FLEX kit, Dako, Agilent - #K800021-2, Santa Clara, CA, USA) according to the manufacturer's instructions. Three semi-serial sections obtained in the sagittal plane from four animals of each experimental group were analyzed. The histological sections were deparaffinized in xylene (Curitiba, Parana, Sao Paulo, rehydrated and subsequently incubated with a 3% hydrogen peroxide solution to block endogenous peroxidase. The sections were subjected to antigen recovery. For the analysis of CD45+ cells (ABclonal #ABclonal # #AB10558 - Woburn, MA, USA) antigen recovery was carried out in a pressure cooker using the Target Retrieval compound from the DAKO kit, with high pH for 3 minutes at 95°C. To identify CD3+ cells (ABclonal #16669 - Woburn, MA, USA), sodium citrate was used in a pressure cooker for 3 minutes at 95°C and then left to cool for 15 minutes. The sections were incubated with primary antibody at a concentration of 1:200 at 4º C in a humidified chamber. For the

CD45 analysis, the primary antibody was used overnight for 16 hours, while for the CD3 analysis, the antibody was incubated for 1 hour. Primary antibody detection was carried out with a biotinylated secondary antibody (N-Histofine #414191F - Chuo-ku, Tokyo, Japan). The reaction was revealed using the chromogen DAB (N-Histofine #425312F - Chuo-ku, Tokyo, Japan). The sections were counterstained with Carrazi's Hematoxylin (St Louis, MO, USA) solution and the coverslips were mounted with Entellan® (Sigma-Aldrich #1.07961.0100 – Burlington, MA, USA. Images were captured using a light microscope (Diastar-Cambridge Instruments - Cambridge Instruments, Buffalo, NY, EUA) at 200X magnification and image capture parameters for brightness, contrast and exposure time were kept constant. The determination of the intensity and number of labeled cells was performed through quantitative analysis by an examiner unaware of the experimental groups using the Aperio software (https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/).

Real-time PCR (qPCR)

Total RNA from gingival tissues was extracted with Trizol reagent (Invitrogen Corp. – Carlsbad, CA, USA) according to the manufacturer's instructions. 700 ng of total RNA was used for cDNA synthesis using random hexamers as primers and following the reagent supplier's instructions (High-capacity cDNA synthesis kit, Applied Biosystems ´- Vilnius, Lithuania). The expression of selected inflammatory genes (IL-6, RANKL and TNF-α – Pleasanton, CA, USA - #433118) was determined by real-time RT-qPCR using TaqMan probes and reagents (TaqMan Fast Gene Expression Assays, TaqMan Universal master mix, Applied Biosystems - Vilnius, Lithuania) in a PCR system StepOne Real Time (Applied Biosystems - Vilnius, Lithuania). For each sample, gene expression analyses were performed in duplicate. To normalize the amount of total cDNA present in each reaction, the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. To compare expression levels between different samples, the relative expression level of genes was calculated using the CT mean comparative method through the thermocycler software.

ELISA Analysis

Total protein was extracted from periodontal tissues using the T-PER extraction buffer (Tissue Protein Extraction Reagent – Pierce Biotechnology – Rockford, IL, USA)

supplemented with a cocktail of protease inhibitors (Protein Stabilizing Cocktail – Santa Cruz – MO, USA). The buffer was added to the tissues, which were macerated for 5 minutes and centrifuged at 13,000 revolutions per minute (RPM) at 4°C with subsequent removal of the supernatant. TNF-α (Northeast Minneapolis, MN, USA) expression was determined using an ELISA kit, according to the manufacturer's instructions (R&D Systems - #RTA00). The concentration of target proteins was normalized to the total protein content determined by the Lowry method (DC assay, Bio-Rad).

Data analyses

Data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The objective of the analysis was to compare the results of each outcome according to the different tested inhibitors. The Shapiro-Wilk test was used to verify that the data had a normal distribution. Pairwise comparisons were performed by unpaired Student t-tests with Welch's correction for unequal variances and by one-way analysis of variance (ANOVA) followed by Tukey test for post-hoc pairwise comparisons. Significance level was set at 95% ($p < 0.05$) in all analysis.

3. RESULTS

There was no loss of animals, no significant changes in body weight, nor any noticeable physical or behavioral changes indicative of adverse effects associated with the experimental protocol.

JAK inhibition prevented bone resorption in ligature-induced periodontitis

μCT three-dimensional analysis by μCT demonstrated that ligature placement of the ligatures induced significant bone resorption in rats and that both JAK inhibitors administered orally significantly reduced ($p<0.05$) alveolar bone resorption in relation to the positive control group (Figure 1A and 1B). The results are representative of analyzes performed with a total number of 7 animals per group.

JAK inhibition prevented pro-inflammatory cell infiltration

A significant reduction of cellular infiltrate in the periodontal tissues of animals treated with JAK1-3i and JAK3i was observes compared to untreated periodontitis animals (positive control group) ($p<0.05$) (Figure 2A). Notably, there was also a significant ($p < 0.01$) increase in collagen content and blood vessels in the gingival tissues of animals treated with the JAK3i compared to the positive control group (Figure 2B and 2C).

Immunohistochemical analysis supported an anti-inflammatory effect for JAK inhibitors indicating a statistically significant reduction in the number of CD45+ cells with JAK1-3i administration ($p<0.05$) (Figure 2D). Interestingly, JAK inhibitors administration did not modulate the increase in CD3+ cells induced by ligatures (Figure 2E). Representative images are in 2F. The results are representative of analyzes performed with a total number of 7 animals per group.

JAK inhibition reduced the expression of inflammatory markers

JAK1-3i and JAK 3 inhibitors administration resulted in a significant reduction in IL-6 and RANKL mRNA levels compared to the negative control ($p<0.05$ (Figure 3A and 3B), while no such reduction was observed for TNF-α. Still, interestingly, there was a difference in TNF-α mRNA expression between JAK1-3i and JAK 3 inhibitors ($p<0.05$) (Figure 3C). When evaluating the effect of JAK inhibition at the protein level of TNF-α by ELISA, a significant reduction of TNF-α was observed for both inhibitors ($p<0.05$) (Figure 3D), suggesting that inhibition of JAK influences the cascade of inflammatory events in the microenvironment associated with periodontitis. The results are representative of analyzes performed with a total number of 5 animals per group for mRNA levels by RT-qPCR analysis and 3 animals per group for protein level by ELISA analysis.

4. DISCUSSION

JAK inhibition through systemic administration of JAK1-3i and JAK3i prevented ligature-induced bone loss in the periodontium and reduced the number of inflammatory cells and the expression of inflammatory markers in the gingival tissue of rats.

Studies have highlighted the relevance of the JAK/STAT pathway in regulating multiple cellular processes essential for bone homeostasis and remodeling (18). In conditions characterized by excessive bone resorption, such as osteoporosis, rheumatoid arthritis, and periodontitis, unbalanced JAK/STAT signaling contributes significantly to the pathophysiology (19). Within the bone microenvironment, cytokines like IL-6, TNF- α, and RANKL orchestrate osteoclast differentiation and activation, leading to bone degradation. JAK activation triggered by these cytokines influences downstream STAT pathways, modulating the expression of genes related to osteoclastogenesis, including c-Fos, nuclear factor of activated T cells cytoplasmic 1, and tartrate-resistant acid phosphatase (20). Moreover, JAK/STAT signaling influences the expression of genes involved in osteoclast activity regulation, such as cathepsin K (20) and matrix metalloproteinases. Therefore, targeting JAK/STAT signaling presents a promising avenue for therapeutic interventions aimed at mitigating bone loss by attenuating excessive osteoclast formation and activity, and connective tissue matrix. Although few studies have evaluated the effect of JAK inhibition/activation on bone turnover cells, data have demonstrated that they actively participate in crucial cellular events such as the proliferation, activation, and survival of osteoclasts and osteoblasts. This interaction has direct implications for diseases resulting in irreversible bone destruction associated with the presence of inflammatory processes, such as periodontitis (21).

The results have provided novel insights on JAK inhibition and its impact on the JAK/STAT signaling pathway in the context of periodontitis and, by extension, other diseases in which this pathway plays a central role. It is estimated that non-surgical periodontal treatment alone fails in approximately 25% of periodontitis cases, resulting in either the arrest or lack of change in progression rates (22). Therefore, the identification of adjunct therapies that can prevent bone loss in people at risk of further periodontitis progression is vital to preserve the dentition and its associated functions, such as mastication and speech. Although studies have already documented the ability of JAK inhibitors, particularly JAK1-3, to prevent inflammation and bone resorption in models of induced arthritis in rats (16,17), the immunological mechanisms underlying the action of these inhibitors are most evidenced in these types of joint lesions, and few results have been demonstrated in other osteolytic disorders, such as periodontitis.

Our results, derived from a study that partly focused on bone resorption during periodontitis induced in rats, align with a previous study conducted on an in vivo model of inflammatory arthritis (16). This arthritis study utilized the JAK1-3i and observed the prevention of bone resorption through JAK inhibition over the same 7-day experimental period as ours. This allowed us to evaluate the effects of modulating the pathway during the progression of inflammation (16). Corroborating other findings in the literature, the reduction in bone resorption associated with JAK inhibition observed in our study was in part explained by a reduction in inflammatory mediators relevant for periodontitis progression as IL-6, RANKL and TNF-α). One of the effects attributed to TNF-α is the stimulation of fibroblasts to generate matrix metalloproteinases, which play a role in degrading elements of the extracellular matrix in both rheumatoid arthritis (23) and periodontitis (24). Additionally, both TNF-α and IL-6 can synergistically collaborate in osteoclastogenesis and enhance osteoclast activity. They act as indirect stimuli for the production of RANKL by other cells, exerting direct effects not only on osteoclast precursor cells but also on mature osteoclasts (25,26). These findings corroborate our findings, as with the reduction of these markers, we also observed prevention of bone resorption and an increase in tissue extracellular matrix (27).

In our study, oral administration of JAK inhibitors at a dose of 6.2 mg/kg showed a significant reduction in the inflammatory infiltrate, increased collagen content, and the formation of new blood vessels within the gingival tissues in a rat model of experimental periodontitis. These results support the theory that selective JAK inhibition can arrest the progression of inflammation and promote tissue repair, a phenomenon previously documented using JAK1-3i (Tofacitinib). This anti-inflammatory effect has been observed in patients with mucosal lesions due to chronic conditions like ulcerative colitis (28,29) and psoriasis, in which administering JAK inhibitors, including those approved by the Food and Drug Administration (FDA), demonstrated reduced tissue inflammation (30,31). Our results also demonstrated an increase in blood vessel proportion by inhibiting JAK, suggesting an attempt to repair the tissue even during disease induction. The literature has demonstrated that the formation of new blood vessels during inflammatory processes is relevant because it provides nutrients and oxygen to regenerate cells, aiding in the resolution of inflammation (32).

We observed that the anti-inflammatory effects of JAK inhibitors were correlated with a decrease in the number of infiltrating inflammatory cells (CD45+), which confirms their anti-inflammatory function since leukocytes are highly expressed in periodontal inflammatory processes and play a crucial role in the immunological response to periodontal infections (33).

In the context of periodontal disease, a lack of comprehensive information concerning JAK expression and its specific role in disease progression remains. A single study examining the effects of JAK3 inhibition in an experimental model of periodontitis presented conflicting results compared to our findings (34). This study reported an increase in inflammatory infiltrate density and alveolar bone loss in mice inoculated with *Porphyromonas gingivalis* (Pg) and administered an oral JAK inhibitor. Moreover, in vitro tests conducted on immune cells stimulated with Pg and treated with the JAK inhibitor demonstrated an elevated production of inflammatory cytokines compared to the control group. It is important to note that the disparities in results might be attributed to the specific experimental model utilized, which encompassed a 43-day experimental period. Additionally, the method used to induce periodontitis differed from the approach adopted in our research, potentially accounting for the divergent outcomes observed in contrast to our data (34).

In the broader context of inflammatory diseases, this research provides significant insights as the results corroborate clinical findings. JAK inhibition has been the subject of clinical studies in several inflammatory diseases, including myelofibrosis (35-39), rheumatoid arthritis (40-46) and periodontitis (42,47). The common point between these diseases is the direct impact on bone tissue as a result of the inflammatory process. Conventional treatments for such conditions often lead to significant side effects in patients, in addition to a high rate of recurrence after completion of treatment. Faced with this challenging scenario, new therapeutic approaches, such as the use of JAK inhibitors, are being thoroughly explored as promising alternatives. The results obtained so far demonstrate significant improvements in the reduction of specific parameters associated with each disease, contributing to improvement in the quality of life of patients who, even after many years of post-treatment follow-up, continue to experience lasting benefits (48).

Limitations in our study must be acknowledged, including the analysis of a single dose of the inhibitors based on previous literature and only 7 days of periodontitis

induction. Despite these limitations, our results suggest that selective inhibition of JAKs may be a promising strategy to modulate inflammatory responses associated with periodontal disease. In view of the results, future studies evaluating the effect of JAK inhibitors in a periodontal repair model and/or the local application may reveal interesting conclusions regarding the effect of JAK inhibition as an adjunct therapy to periodontitis.

5. CONCLUSION

Inhibition of the JAK pathway through inhibitors targeting JAK 1-3 and JAK 3, demonstrated a significant impact on preventing the inflammatory destruction in bone and gingival tissues in a model of experimentally induced periodontitis. These findings provide a biological basis for the development of adjunctive therapies that are more effective for patients who do not respond adequately to periodontal therapy.

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Conflict of Interest. None to declare.
Ethics Approval Statement. This experimental protocol was approved by the Animal Use Ethics Committee (CEUA) of the Faculty of Dentistry of Araraquara - UNESP (process 27/2021).
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Figures:

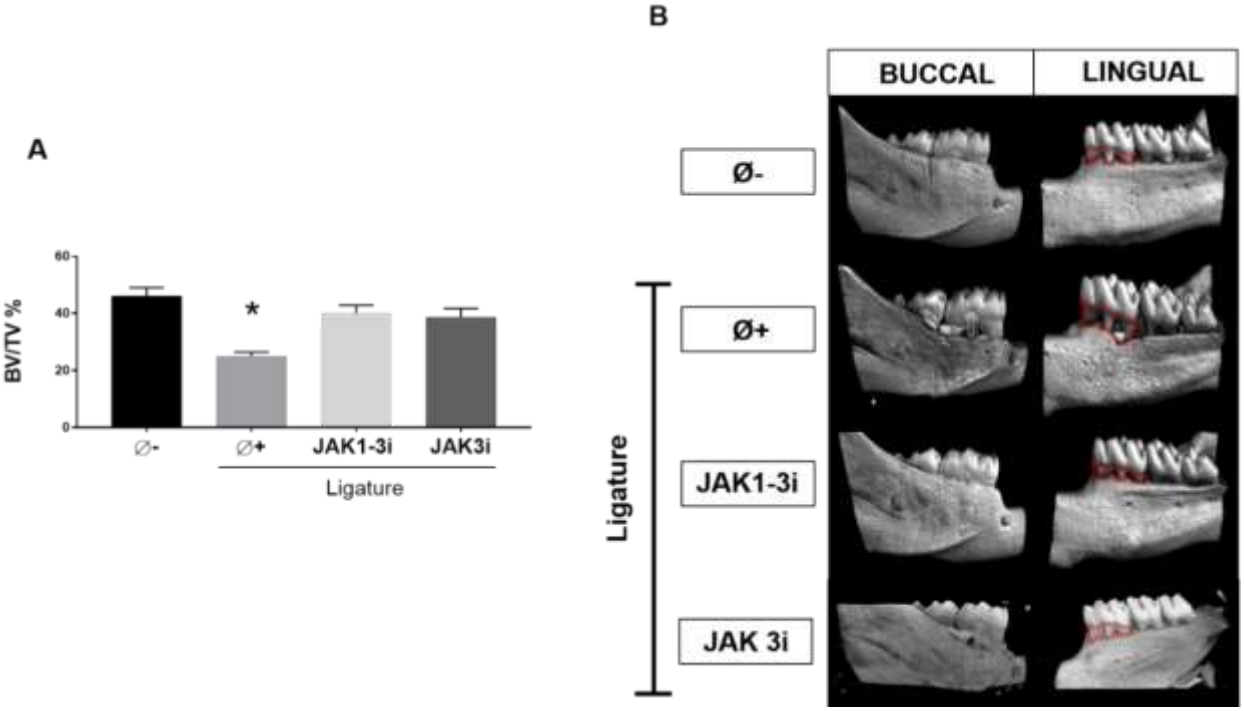


Figure 1. JAK inhibition demonstrated a positive effect in preventing ligature-induced bone resorption under concomitant administration of JAK1-3 and JAK3 inhibitors when compared to negative control group (Ø-) (A). Representative images of each experimental group were obtained in 3D planes (B). - *$p<0.001$ in relation to other groups. The bars indicate mean values while the error bars indicate standard error of the mean.

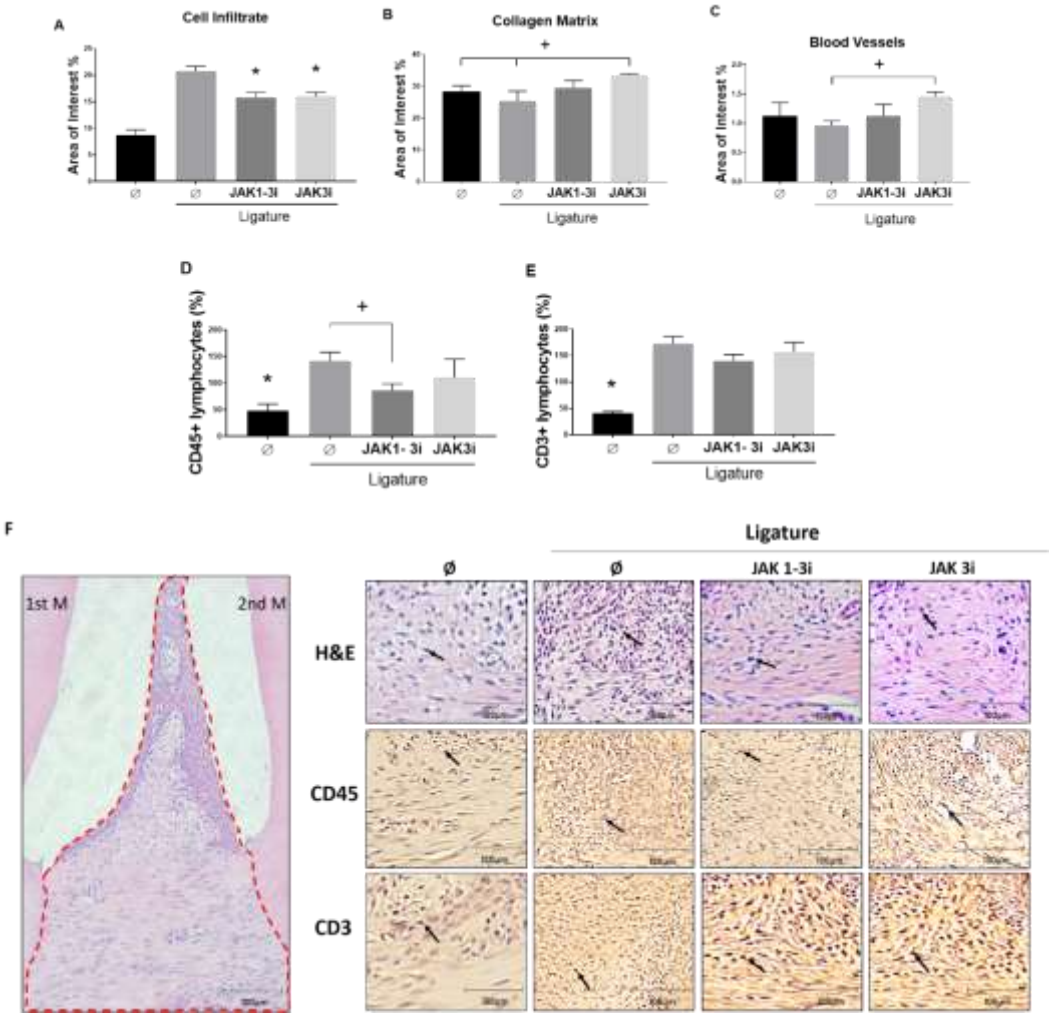


Figure 2. JAK inhibition prevented inflammation in gingival tissues during experimental periodontitis in rats. Stereometric analysis of cellular infiltrate: JAK1-3i and JAK3i groups presented decreased cellular infiltrate (A); Stereometric analysis of collagen matrix: JAK3i showed increased collagen matrix (B); Stereometric analysis of blood vessels: JAK3i increased the number of blood vessels (C). JAK1-3i inhibition led to reduced staining of CD45 in the region of interest but not CD3. Percentage of cells stained positive with antibodies to CD45 (D). Percentage of cells stained positive with antibodies to CD3 (E). Representative histological images used for stereometric and immunohistochemical analysis (400x magnification), arrows indicate inflammatory infiltrate cells in the H&E-stained images and positive staining for immunohistochemistry. (F). The bars indicate the percentage mean value and the error bars indicate the standard error of the mean (SEM); *$p<0.05$ compared to the other groups, +$p<0.05$ between the indicated groups. 1st M: Mandibular First Molar. 2nd M: Mandibular Second Molar.

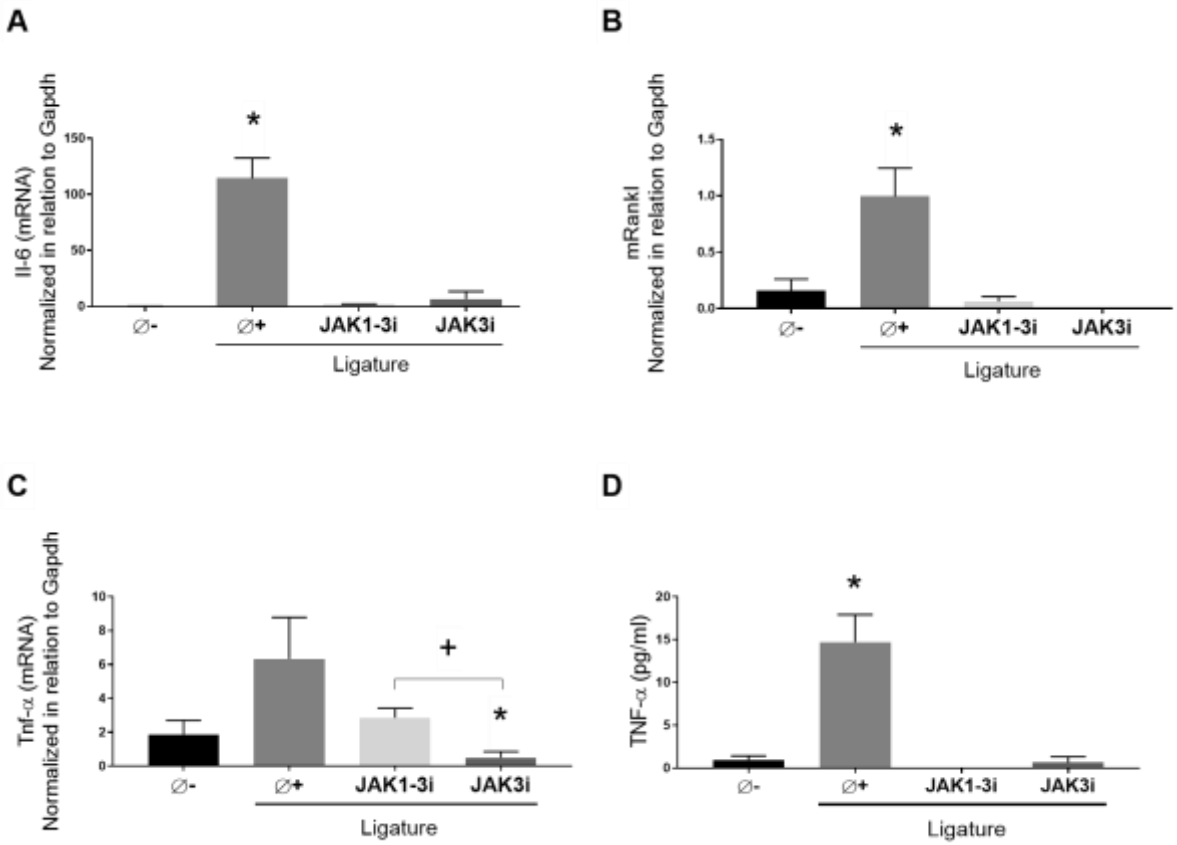
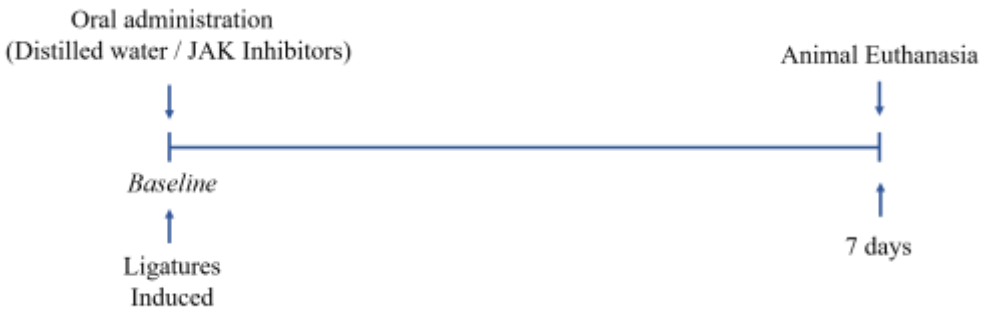


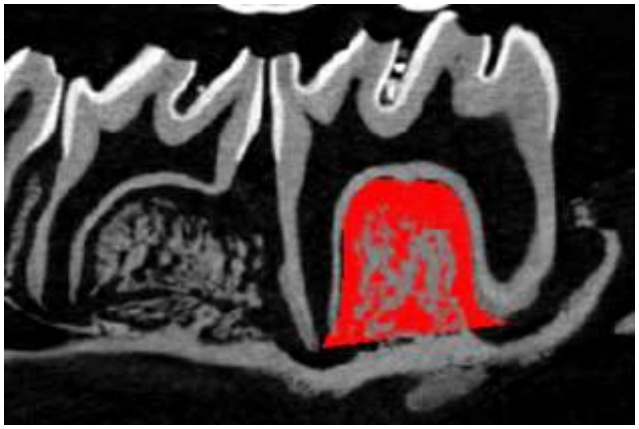
Figure 3. JAK inhibition led to decreased expression of inflammatory indicators in gingival tissues with periodontal disease. From the RT-qPCR experiment, a significant reduction in IL-6 (A) and RANKL (B) gene expression was observed with both JAK inhibitors, without changes in TNF-α levels (A). A difference in TNF-A mRNA expression was only observed between the groups of animals treated with the inhibitors. However, based on the ELISA analysis, there was a significant reduction in TNF-α with both JAK inhibitors (D). The bars indicate the percentage mean value and the error bars indicate the standard error of the mean (SEM), *$p<0.05$ compared to the other groups, +$p<0.05$ between the indicated groups.

Supplementary Table 1. Experimental groups (n=40) according to the compound administered and the induction of periodontitis.

Groups	Periodontitis Induction	Procedure (Systemic Administration)
Negative Control (Ø-)	No	Distilled water
Positive Control (Ø+)	Yes	Distilled water
JAK1-3i	Yes	JAK1-3 Inhibitor (6.2mg/kg)
JAK3i	Yes	JAK3 Inhibitor (6.2mg/kg)



Supplementary figure 1. Timeline demonstrating the experimental period, administration of distilled water and JAK inhibitors, as well as the euthanasia period.



Supplementary figure 2. Representative image of the standardized region of interest (ROI), defined in sagittal orientation in the furcation area of the lower first molar, extending from the top of the furcation to the root apices.

4 CONCLUSÃO

A inibição de JAK, utilizando os inibidores farmacológicos das isoformas de JAK 1-3 e JAK 3, teve um impacto significativo na redução das alterações inflamatórias, tanto no tecido ósseo, quanto gengival dos animais com periodontite experimentalmente induzida, além de prevenir a diferenciação de osteoclastos e liberação de ROS, e favorecer a proliferação e mineralização óssea *in vitro.* A inibição de JAK3 demonstrou um efeito mais eficaz na modulação do processo inflamatório em alguns desfechos avaliados *in vitro*. Estes resultados indicam a relevância da via JAK-STAT na patogênese da periodontite, e sugerem que a modulação negativa de JAK pode ser uma alternativa terapêutica promissora no tratamento adjunto da doença.

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APÊNDICE A – METODOLOGIA

ESTUDO IN VIVO

Este protocolo experimental foi aprovado pelo Comitê de Ética em Uso Animal (CEUA) da Faculdade de Odontologia de Araraquara - UNESP (processo 27/2021) e foi realizado de acordo com as diretrizes do Conselho Nacional de Controle da Experimentação Animal do Colégio Brasileiro de Experimentação Animal (CONCEA).

Animais

Foram utilizados 40 ratos *Holtzman* (Rattus norvegicus albinus, *Holtzman*), machos, pesando entre 100-200g, que foram mantidos em gaiolas de polipropileno (5 animais/gaiola) com temperatura (21 ± 1C) e umidade (65-70%) controlada e um ciclo claro-escuro de 12 horas. Os animais receberam alimentação granulada (Labina / Purina) e água *ad libitum.*

Efeito da inibição de JAK sobre a progressão da periodontite

Para avaliarmos o efeito da inibição de JAK durante o desenvolvimento da doença periodontal, os animais foram aleatoriamente divididos em 4 grupos experimentais (10 animais por grupo), como descrito no Quadro 1. O cálculo do tamanho da amostra foi baseado em nossos dados prévios, que tiveram como desfecho primário a análise do volume ósseo por microtomografia óssea em ratos[1]. Adotando-se um poder β do estudo de 0.8 e o poder α de 0.05, determinou-se uma amostra mínima de 7 animais por grupo.

Quadro 1- Grupos experimentais (n=40) no modelo de progressão, de acordo com o composto administrado e presença de doença periodontal

Grupos	Indução da Doença Periodontal	Procedimento (Administração Sistêmica)
Controle Negativo	Não	Água Destilada
Controle Positivo	Sim	Água Destilada
JAK1-3i	Sim	Inibidor de JAK1-3 (6,2mg/kg)
JAK3i	Sim	Inibidor de JAK3 (6,2mg/kg)

Fonte: Elaboração própria.

Os animais foram submetidos à indução da doença periodontal através da instalação de ligaduras ao redor dos primeiros molares, bilateralmente. Para isso os animais foram anestesiados (0,08 mL de quetamina e 0,04 mL de cloridrato de xilazina por 100 g de peso corporal) e posicionados em mesa operatória para instalação das ligaduras (fio de algodão número 24), que ficaram mantidas em posição durante os 7 dias do período experimental.

Para avaliarmos o efeito da seletividade dos inibidores sobre os resultados, foram administrados dois tipos de inibidores: um inibidor seletivo para isoformas de JAK1-3 (CP-690-550, Cayman), e um inibidor específico para isoforma de JAK3 (TL6-144). Diversos inibidores de JAK1-3 foram aprovados pela FDA e têm sido usados no tratamento de artrite reumatoide, psoríase e outras condições inflamatórias, mostrando benefícios na redução dos sinais e sintomas clínicos destas doenças. No presente estudo usaremos um inibidor de JAK1-3 obtido comercialmente (Cayman). O inibidor seletivo para isoforma de JAK3, utilizado neste estudo, foi sintetizado pelo laboratório do Prof. Dr Nathanael Gray, Stanford University, Estados Unidos, colaborador neste projeto. Considerando a função essencial de JAK3 na sinalização da resposta imune, e sua expressão restrita em tecidos hematopoiéticos, Dr. Gray tem trabalhado na síntese de inibidores seletivos para JAK3 como uma estratégia para avaliar o papel desta isoforma em diferentes doenças imunes e no câncer. Resultados da análise farmacológica do inibidor (TL6-144), demonstraram ausência de efeitos secundários associados a administração oral e inibição efetiva de JAK-3 em

murinos[2,3]. Seu impacto sobre a inflamação e perda óssea em modelo experimental de periodontite in vivo foi avaliado pela primeira vez neste estudo.

Os animais receberam a administração diária intragástrica dos inibidores (6,2mg/kg) durante 7 dias. Após esse período todos os animais foram eutanasiados. A dose dos inibidores foi baseada em estudos utilizando modelo experimental de artrite reumatoide induzida por adjuvante, que demonstraram uma redução significativa do infiltrado inflamatório na articulação de animais tratados após 4 e 7 dias de administração oral, sem causar efeitos adversos[4,5]. Animais do grupo controle negativo e positivo receberam água destilada.

Os procedimentos delineados dentro do período experimental estão representados na figura 1.

Figura 1 - Linha do tempo demonstrando o período experimental, a administração da água destilada, e dos inibidores de JAK, bem como o período de eutanásia

Fonte: Elaboração própria.

Obtenção das amostras

No momento da eutanásia, o maxilar inferior foi dividido em duas hemimandíbulas. O lado esquerdo foi fixado em paraformaldeido 4% à 4C por 18h, transferido para etanol 70% e armazenado à 4°C. Estas amostras foram utilizadas na avaliação imunohistoquímica, identificação de osteoclastos e estereometria. A hemimandíbula direita foi utilizada para microtomografia computadorizada, e os tecidos periodontais não mineralizados ao redor dos primeiros molares inferiores de cada animal foram dissecados cuidadosamente e processados para as análises moleculares (avaliação dos níveis proteicos por ensaio ELISA, e mRNA por RT-qPCR, segundo descrito na seção correspondente).

Adicionalmente, no momento prévio à eutanásia, os animais foram anestesiados e foi coletado sangue para contagem das células sanguíneas (série branca) de cada animal.

Avaliação da perda/reparo óssea por microtomografia computadorizada

As hemimandíbulas foram reduzidas com discos diamantados montados em mandril e peças de baixa rotação de forma a incluir apenas os primeiros e segundos molares inferiores. As peças foram digitalizadas em um sistema de imagem por μCT (Skyscan 1076; Bruker, Kontich, Bélgica) usando resolução espacial isotrópica de 9 μm (0,01 mm), filtro de alumínio de 0,5 mm, tensão de 80 kV, corrente de 310 μA, exposição de 195 ms, média de quadro de 03 e passo de rotação de 0,4° em 180°. Para reconstrução (NRecon 1.6.1.5; Bruker), foram utilizados os seguintes parâmetros: correção de artefato em anel, 4; máscara de defeito de pixel, 5%; alisamento, 1%; correção de endurecimento do feixe, 26% e sensor comprimido (CS) para conversão de imagem, 0,0-0,8. A região de interesse padronizada (ROI) foi definida em uma orientação sagital na área de furca do primeiro molar inferior, estendendo-se do topo da furca até os ápices radiculares. Esse ROI foi delimitado a cada dez planos, com 90 fatias. A fração do volume do ROI ocupado pelo tecido mineralizado (volume da fração óssea (BVF)) foi quantificado. A análise foi realizada por um examinador experiente e cego para os grupos experimentais.

Processamento histológico

As hemimandíbulas, incluindo os primeiros e segundos molares inferiores, foram descalcificados em solução de EDTA 10%, pH 7.2 por 6 a 8 semanas para posterior preparo e inclusão em parafina. Para as análises de imuno-histoquímica foram obtidos cortes semi-seriados de 4 μm, montados em lâminas silanizadas. Para as análises de identificação de osteoclastos e estereometria foram utilizados cortes semi-seriados de 4 μm de espessura em lâminas de vidro comuns, coradas com hematoxilina e eosina (H/E).

Imunohistoquímica – Expressão de CD45 e CD3

A presença de processo inflamatório (representado pela quantidade de leucócitos - células CD45+ e linfócitos T - CD3+) foi identificada e quantificada nos

tecidos gengivais dos diferentes grupos experimentais na região entre primeiros e segundos molares inferiores. As análises foram realizadas pelo método biotina-estreptavidina-HRP-DAB (kit Envision FLEX, Dako, Agilent - #K800021-2) de acordo com as instruções do fabricante. Três cortes semi-seriados obtidos no plano sagital plano (4 µm de espessura, abrangendo uma distância de 320 µm) de quatro animais de cada grupo experimental foram analisados. Os cortes histológicos foram desparafinizados em xilol, rehidratados e posteriormente incubados com solução de peróxido de hidrogênio 3% para bloqueio da peroxidase endógena. Os cortes foram submetidos à etapa de recuperação antigênica, sendo que para a análise de CD45 (ABclonal #10558) utilizamos uma recuperação em panela de pressão utilizando o composto Target Retrival do kit DAKO com pH alto por 3 minutos à 95°C, e para análise de CD3 (ABclonal #16669) utilizamos o citrato de sódio (preparado no próprio laboratório) de pH baixo, também em panela de pressão, pelo mesmo período de tempo e mesma temperatura, e então deixados sobre a bancada para resfriamento por mais 15 minutos. A atividade da peroxidase endógena foi inibida pela adição de peróxido de hidrogênio 3% por 15 minutos. Em seguida os cortes foram incubados com anticorpo primário em uma concentração de 1:200 a 4º C em um compartimento umidificado. Para a análise de células CD45+ o anticorpo primário foi deixado sobre os cortes overnight (16 horas), enquanto para a análise de CD3+, o anticorpo foi incubado por apenas 1 hora. A detecção do anticorpo primário foi feita com anticorpo secundário biotinilado (N-Histofine # 414191F) e após incubação em solução de streptavidina conjugada à peroxidase (streptavidin-HRP) a reação foi revelada com o uso do cromógeno DAB (N-Histofine #425312F). Os cortes foram contra-corados com Hematoxilina de Carrazi e em seguida as lamínulas foram montadas com Entellan® (Sigma #1.07961.0100). As imagens foram capturadas em microscópio de luz (Diastar-Cambridge Instruments) na magnificação de 200X e parâmetros de captura da imagem (brilho, contraste, tempo de exposição) mantidos constantes. A determinação da intensidade e número de células marcadas foi realizada através de análise quantitativa, por um examinador cego para as condições experimentais, usando o software Aperio (https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/).

Análise morfométrica e estereométrica

Sete dias após a colocação das ligaduras, os animais foram eutanasiados e as mandíbulas foram divididas em duas hemimandíbulas. Posteriormente, as hemimandíbulas foram fixadas com paraformaldeído a 4% por 18 horas e posteriormente, descalcificadas em EDTA 0,5% (pH 7,2) por 3 meses, embebidas em parafina e utilizadas para análise histológica. Os cortes histológicos foram corados com H/E, e as imagens obtidas em microscópio óptico convencional utilizando configurações padronizadas para aquisição de imagens (Leica Application Suite 3.8, Wetzlar, Alemanha). A quantificação de osteoclastos, identificados pela morfologia e localização, foi realizada com auxílio de microscópio de luz (Leica DM2500) com ampliação de 200x. Foram considerados osteoclastos, células multinucleadas, em proximidade com a superfície óssea, localizadas em lacunas de reabsorção entre primeiros e segundos molares inferiores, e região de furca do primeiro molar. Os resultados foram utilizados para determinar um valor médio por animal e depois esses valores foram comparados entre os grupos.

Para análise estereométrica do processo inflamatório, a proporção de células (células fibroblásticas, células inflamatórias ou não definidas) e fibras colágenas na área de interesse foi determinada como a porcentagem do total de pontos da grade efetivamente contados. Para isso, foi determinada uma região de interesse para cada corte, delimitada pelas seguintes estruturas anatômicas: coronalmente pela porção mais apical do tecido epitelial; apicalmente pelo topo da crista óssea; e lateralmente pela região mais proeminente da raiz distal do primeiro molar e pela raiz mesial do segundo molar. As imagens foram obtidas utilizando um microscópio óptico acoplado a uma câmera digital colorida, com configurações padronizadas para aquisição de imagens (Leica Application Suite 3.8, Wetzlar, Alemanha), com ampliação de 200x. Após a captura das imagens, a quantificação foi realizada por meio de grades posicionadas (sobrepostas) nas imagens digitalizadas dos cortes histológicos, preenchendo toda a região de interesse da imagem. Para cada corte foram necessárias duas grades, cada uma contendo 90 pontos de intersecção, e a proporção de componentes teciduais na área de interesse foi obtida em relação ao número total de pontos contados. Foram analisadas pelo menos 3 seções por animal, de pelo menos 4 animais por grupo. Todas as análises foram realizadas por um examinador treinado e cego para os grupos experimentais.

ELISA

Amostras de proteína total foram extraídas dos tecidos periodontais removidos no momento da eutanásia, utilizando o tampão de extração T-PER (Tissue Protein Extraction Reagent – Pierce Biotechnology,), suplementado com um coquetel de inibidores de protease (Protein Stabilizing Cocktail – Santa Cruz). O tampão foi adicionado aos tecidos, os quais foram macerados por 5 minutos a 13.000 rotações por minuto (RPM) a 4°C e centrifugados com posterior remoção do sobrenadante. As amostras de proteína foram utilizadas para determinarmos a expressão de TNF alpha, através do kit ELISA, de acordo com as instruções do fabricante (R&D Systems). A concentração das proteínas-alvo foi normalizada para o conteúdo de proteína total, determinado pelo método de Lowry (DC assay, Bio-Rad).

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

RNA total dos tecidos gengivais foram extraídos com o reagente Trizol (Invitrogen Corp.) de acordo com as instruções do fabricante. 700 mg de RNA total foram utilizados para a síntese de cDNA utilizando random hexamers como primers e seguindo as instruções do fornecedor dos reagentes (High capacity cDNA synthesis kit, Applied Biosystems). A expressão dos genes inflamatórios selecionados (Il-6, Rankl e Tnf-α) foi determinada por RT-qPCR tempo real usando sondas e reagentes Taqman (TaqMan Fast Gene Expression Assays, TaqMan Universal master mix, Applied Biosystems) em um sistema de PCR Tempo Real StepOne (Applied Biosystems). Para cada amostra, as análises da expressão gênica foram realizadas em duplicata. Para normalizar a quantidade de cDNA total presente em cada reação, a expressão de GAPDH, que não é alterada pelas condições experimentais, foi usada como controle endógeno por ser um gene constitutivo. Para comparar os níveis de expressão entre as diferentes amostras, o nível de expressão relativa dos genes foi calculado usando o método comparativo CT mean utilizando o software da termocicladora.

Contagem Sanguínea - Hemograma

Ao fim do período experimental, os animais foram anestesiados e amostras de sangue venoso periférico foram coletadas por punção intracardíaca, para contagem

das células sanguíneas (série branca: leucócitos, neutrófilos, monócitos plaquetas e linfócitos) de cada animal.

ESTUDO IN VITRO

Inibição de JAK sobre a diferenciação de osteoclastos – Coloração do anel de Actina (microscopia de fluorescência)

Macrófagos murinos (RAW 264.7) foram utilizados como células precursoras de osteoclastos. As células foram plaqueadas (2 x 10^3 células/poço), em placa de 96 poços, e cultivadas em α-MEM suplementado (10% FBS + 1% P/S) na presença de RANKL (100 ng/ml) para diferenciação dos macrófagos em osteoclastos. Diferentes concentrações dos inibidores de JAK (0,5;1 e 2 µg/ml), ou veículo (DMSO 1%), foram acrescentados ao meio de cultura, em concentrações não citotóxicas, 30 minutos antes do estímulo com RANKL. O meio de cultura contendo o composto e o RANKL foram substituídos em intervalos de 48h, retirando-se 100 µL com o auxílio de uma micropipeta e adicionando o mesmo volume de meio novo com os tratamentos propostos. As culturas foram observadas diariamente em microscópio invertido até o término do experimento. Ao final do período experimental, as células foram fixadas e permeabilizadas (BD Cytofix/Cytoperm, BD Biosciences). Em seguida, as células foram coradas com Faloidina conjugada com Alexa Fluor 488 (40 min à temperatura ambiente) e subsequentemente com DAPI (0.5 ug/mL, 5 min à temp. ambiente) e visualizadas em microscópio invertido de fluorescência (Evos fl, AMG Micro) na objetiva de 10x. O número de osteoclastos foi quantificado em toda a extensão do poço, sendo considerado osteoclastos células com 3 ou mais núcleos. O experimento foi realizado três vezes em triplicata de maneira independente.

Produção de ROS (Citometria)

A produção de espécies reativas de oxigênio (ROS) foi avaliada por meio de ensaio utilizando substrato solúvel absorvido passivamente por células vivas, que sofre oxidação na presença de espécies de oxigênio reativas presentes no citoplasma, e passa a emitir fluorescência quando excitado por luz no comprimento de onda de 488 nm (Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit I36007). Brevemente, os macrófagos foram plaqueados com uma quantidade de 1x10^5

células/poço em placas de 96 poços. Após adesão, as células foram estimuladas com 25 µM carboxy-H2 DCFDA por 30 minutos previamente ao tratamento com doses não citotóxicas dos inibidores de JAK, ou veículo (DMSO). Após 30 minutos do tratamento com os inibidores, as células foram estimuladas com LPS (1 µg/mL) por 5 minutos. Por fim, o composto Hoechst 33342 foi adicionado sobre as células em uma concentração final de 1,0 µM para a solução de coloração carboxi-H2 DCFDA durante os últimos 5 min minutos da incubação. As células foram então destacadas enzimaticamente utilizando e analisadas em citômetro de fluxo (FACS Verse, BD Biosciences) para avaliação da proporção de células emitindo fluorescência (i.e., produzindo ROS). A intensidade da fluorescência é proporcional à quantidade de ROS produzida na célula e foi detectada no canal FL2 (FITC) de citômetro de fluxo.

Avaliação da expressão gênica de citocinas (RT-qPCR)

Células RAW 264.7 serão plaqueadas ($3x10^5$ células/poço) em placa de 24 poços e cultivadas em DMEM completo na presença de RANKL (100 ng/mL). Para investigar o efeito do composto na expressão de marcadores relacionados à osteoclastogênese, diferentes concentrações não citotóxicas de JAK1-3I e JAK3 serão adicionadas ao meio de cultura, 30 min antes da estimulação com RANKL, e mantidas por 3 e 5 dias. O meio de cultura contendo o composto e/ou RANKL será substituído em intervalos de 48h. Ao final do terceiro e quinto dia, o RNA total será isolado das células com o kit Purification (Cellco Biotec), conforme instruções do fabricante. A quantificação e pureza das amostras de RNA serão realizadas em espectrofotômetro de nanovolume (Nanoview Plus, GE Healthcare, Buckinghamshire, Reino Unido) e 500 ng serão transcritos reversamente em cDNA (kit de síntese de cDNA de alta capacidade, Applied Biosystem). A detecção da expressão gênica na amostra será realizada utilizando o sistema TaqMan Fast Universal PCR Master Mix (Applied BiosystemsTM; Thermofisher Scientific, Waltham, MA, EUA). A PCR será realizada para detectar os genes alvo de interesse (Nfatc1, receptor semelhante à imunoglobulina associado a osteoclastos (Oscar), fosfatase ácida 5, resistente ao tartarato (Acp5), Mmp-9 e Ctsk) e a expressão de gliceraldeído- A 3-fosfato desidrogenase (Gapdh) será utilizada para normalizar os resultados, como um gene constitutivo não afetado pelas condições experimentais. Para comparar os níveis de expressão entre diferentes amostras, o nível de expressão relativa dos genes será

calculado usando o método comparativo Δ (ΔCT) usando o software do termociclador. Os períodos de 3 e 5 dias foram selecionados a partir dos experimentos preliminares avaliando os períodos de maior expressão dos genes alvo após estimulação com RANKL.

Migração Celular

Para avaliar o efeito dos inibidores de JAK sobre a migração de fibroblastos gengivais de ratos, será utilizado o método Scratch, baseado na criação de uma interrupção de continuidade de uma monocamada celular, ou ferida, e o acompanhamento do fechamento desta ferida por observação em microscópio invertido de fase. Esta investigação será desenvolvida com o uso de cultura celular primária através da extração de fibroblastos gengivais de ratos machos Holtzman (150-200g), a partir de um fragmento de tecido gengival. Após a coleta, o tecido gengival será colocado em meio de cultura completo DMEM contendo 1% de solução antibiótica e antimicótica (Gibco Antibiotic-Antimycotic - 10.000 unidades/mL de penicilina, 10.000 ug/mL de estreptomicina e 25 ug/mL de Gibco Anfotericina B; Gibco, Carlsbad, CA, EUA), sem soro fetal bovino (FBS - Gibco, Carlsbad, CA, EUA). Em seguida, o fragmento de tecido será dissociado fisicamente por meio de bisturi e posteriormente submetido à dissociação enzimática com colagenase P (Sigma Aldrich - Mannheim, Germany), concentração de 2 mg/mL, em DMEM livre de soro a 37 °C e 5% CO_2. Após 24h, as células serão coletadas, centrifugadas e transferidas para um novo frasco de cultura celular (75cm - Corning, Nova York, NY, EUA) contendo DMEM completo + 10% de FBS. Após a proliferação, 5 x 10^5 células serão cultivas em placa de 60 mm, e incubadas durante 24 horas em estufa de CO_2 a 37ºC. Com constatação da confluência da monocamada, o Scratch, ou seja, a ferida, será produzida com a confecção de uma linha reta utilizando uma ponta de pipeta de 200µL. A seguir, os debris formados serão removidos, lavando-se a placa uma vez com 1ml de meio fresco e em seguida, as placas receberão 2ml de meio fresco (Grupo controle) ou meio contendo doses não citotóxicas dos inibidores de JAK. Após 30 minutos as células serão estimuladas ou não com LPS. O teste de migração será realizado nos tempos 0, 12, 24 e 48 horas. As imagens serão capturadas, nos períodos citados, com câmera digital acoplada ao microscópio invertido de fase, e sempre do mesmo campo de visão da ferida, criando pontos de referência na parte externa da placa e na platina

do microscópio com marcadores de ponta fina. Posteriormente, as imagens serão analisadas quantitativamente usando identificação do núcleo celular, o que permitirá a contagem do número de células presentes na área da ferida, previamente definida, obtendo-se a quantidade de célula por área (Índice de Migração Celular) em cada tempo experimental.

Efeito da inibição de JAK sobre atividade de fibroblastos gengivais

A cultura primária de fibroblastos gengivais de ratos será utilizada para essa investigação. Fibroblastos estimulados podem produzir uma série de mediadores inflamatórios capazes de agir direta e indiretamente sobre a degradação de tecido conjuntivo e ósseo. O objetivo deste experimento é avaliar se a inibição de JAK é capaz de reduzir a expressão de genes pró-inflamatórios (Il-6, Il-10, Il-1β, Tnf-alpha, Il-17, Cola1a) por fibroblastos gengivais, como um mecanismo de ação inibitória sobre a inflamação e osteoclastogênese.

Efeito da Inibição de JAK na formação de nódulos de mineralização - Vermelho de Alizarina

MC3T3-E1, células pré-osteoblásticas de camundongos (Sigma-Aldrich, San Luis, MO, EUA) foram plaqueadas em uma placa de 96 poços, com 3 x 10^3 células por poço, e cultivadas com α-MEM suplementado (10% FBS + 1% P/S), com diferentes concentrações não citotóxicas dos inibidores de JAK (0.5, 1, 2 e 5 µg/mL), determinadas em experimentos preliminares, por 14 dias. Após o período, o meio foi aspirado e os poços lavados com PBS (Invitrogen, Carlsbad, CA, EUA). As células foram fixadas com paraformaldeído a 4% por 60 min a 4°C, lavadas com água destilada e coradas com solução de vermelho de alizarina a 2% (SigmaAldrich, San Luis, MO, EUA). por 15 min. Após a remoção do corante, cloreto de cetilpiridínio 10% (Sigma-Aldrich, San Luis, MO, EUA) foi adicionado por 10 min para solubilizar os nódulos de cálcio corados com vermelho de alizarina, e a solução foi transferida para uma nova placa, de 96 poços, para leitura em espectrofotômetro à 550 nm.

Inibidores de JAK aumentam a proliferação de células pré-osteoblásticas MC3T3-E1

As células pré-osteoblásticas de camundongos MC3T3-E1 (Sigma-Aldrich, San Luis, MO, EUA) foram plaqueadas em uma placa de 96 poços, com 3 x 10^3 células por poço, e cultivadas com α-MEM suplementado (10% FBS + 1% P/S), com

diferentes concentrações dos inibidores de JAK (0.5, 1, 2 e 5ug/mL), por 1, 3 e 7 dias. Após os períodos as células foram contadas com auxílio de azul de tripan para avaliar o aumento ou diminuição da proliferação celular.

Análises de dados

Os dados obtidos foram analisados usando GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, EUA). As análises estatísticas tiveram como objetivo comparar os resultados de acordo com as diferentes condições experimentais em cada período. Após análise da distribuição normal pelo teste de Shapiro-Wilk, os dados foram analisados pelo teste paramétrico ANOVA One-Way para amostras independentes ou teste não paramétrico de Kruskal-Wallis, seguido pelos testes post hoc de Tukey ou Dunn para comparações entre pares, respectivamente.

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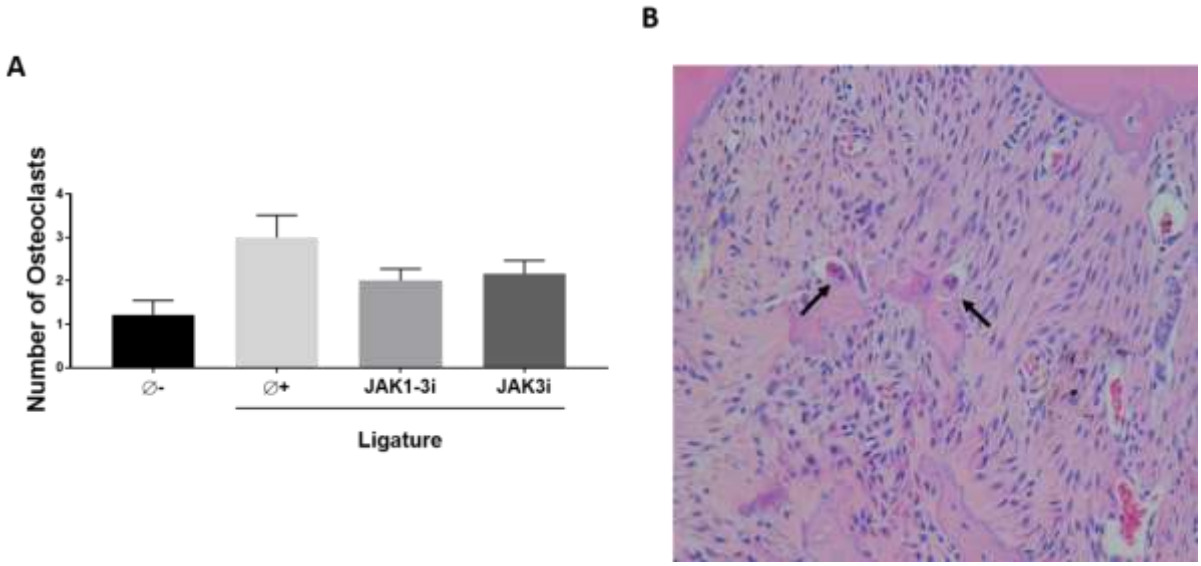
APÊNDICE B - RESULTADOS ADICIONAIS

Estudo in vivo

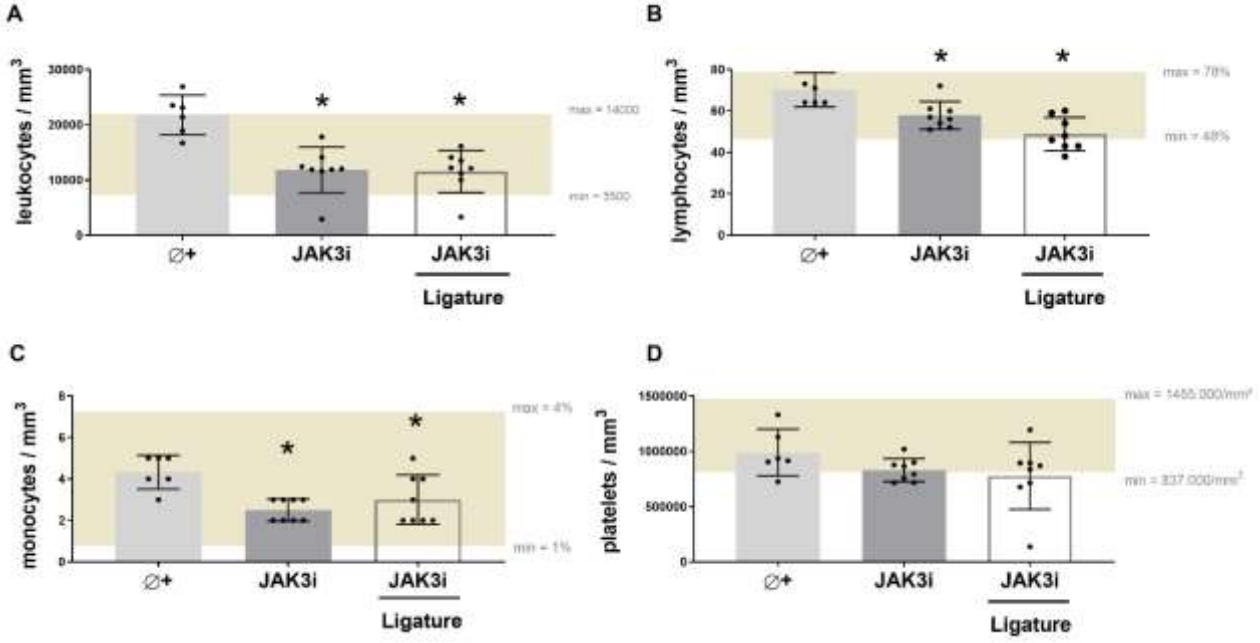
Figura adicional 1 - Inibição de JAK não altera significativamente o número de osteoclastos



Embora os inibidores de JAK tenham prevenido a reabsorção óssea induzida pela colocação das ligaduras, o número de osteoclastos não foi modificado. Cortes histológicos dos animais tratados com inibidores de JAK apresentaram uma discreta redução no número de osteoclastos, em comparação ao grupo controle positivo, entretanto, essa redução não foi estatisticamente significante (Figura adicional 1A). Células gigantes multinucleadas, em proximidade da crista óssea da região de furca do primeiro molar, foram consideradas para a quantificação dos osteoclastos. Em (Figura adicional 1B) o aspecto histológico dos osteoclastos. As barras indicam valores médios e as linhas verticais indicam o erro padrão da média.

Fonte: Elaboração própria.

Figura adicional 2 - A inibição de JAK3 reduziu sistemicamente o número de leucócitos

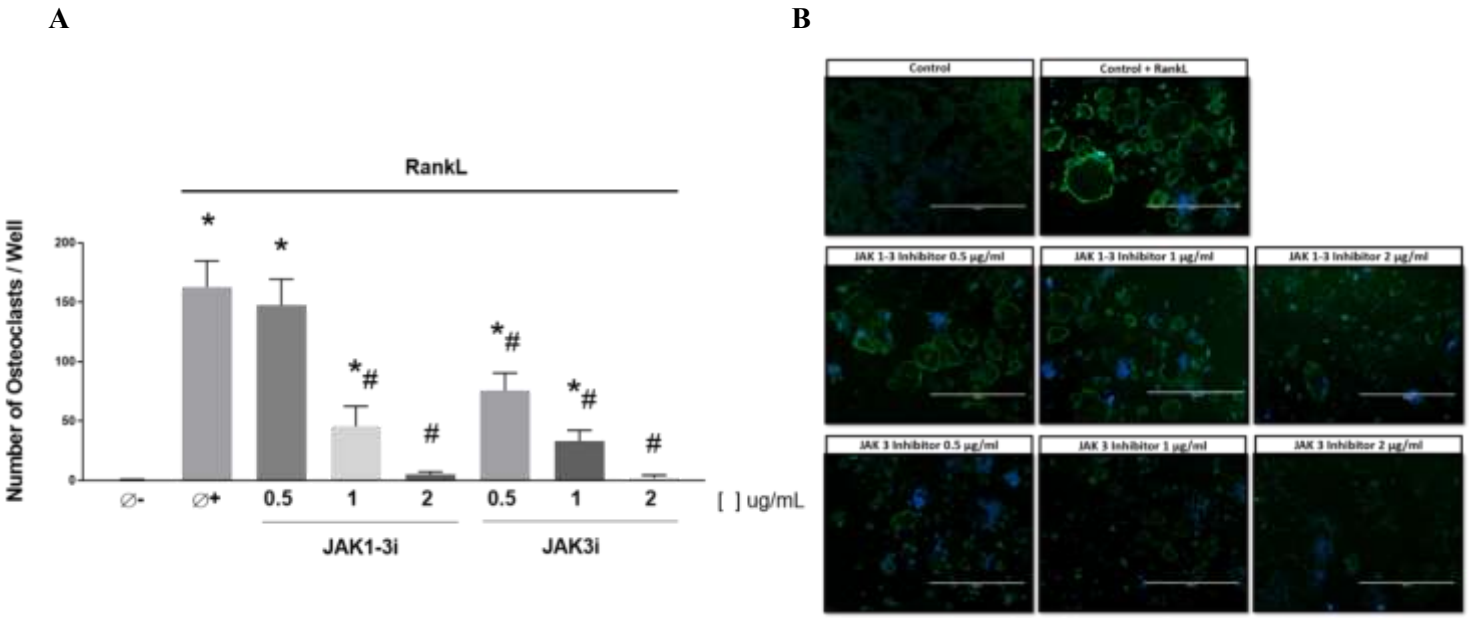


Após sete dias da indução da periodontite e administração do inibidor de JAK3, o sangue periférico dos animais foi coletado por punção cardíaca para contagem das células sanguíneas (leucócitos totais, neutrófilos, monócitos, linfócitos e plaquetas). A análise dos resultados indicou que o inibidor de JAK3 reduziu marcadamente o número de todos os tipos celulares avaliados, em relação ao grupo controle positivo, com exceção do número de plaquetas (Figura adicional 2A, 2B, 2C, 2D). O número de células em cada grupo, entretanto, não diferiu dos animais não tratados (controle negativo). A faixa bege indica a referência do número máximo e mínimo de cada tipo celular em animais da mesma espécie. *p<0.05 entre os grupos identificados e o grupo Ø+. ANOVA seguida de teste post-hoc de Tukey.

Fonte: Elaboração própria.

Estudo in vitro

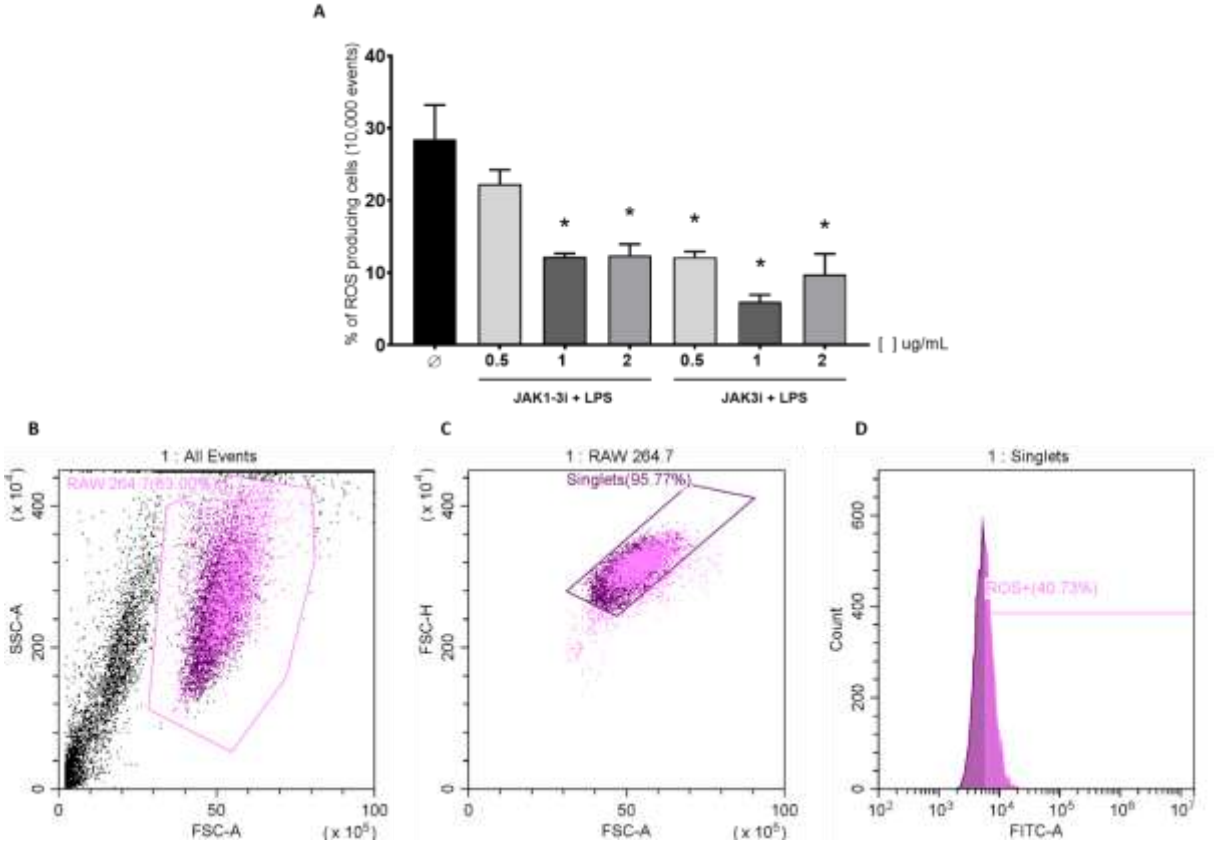
Figura adicional 3 - Os inibidores de JAK 1-3 e JAK 3 reduzem a diferenciação de osteoclastos induzida por RANKL *in vitro,* de maneira dose-dependente.



Células RAW 264.7 foram tratadas com inibidores de JAK1-3 e JAK3 e após 30 min estimuladas com RankL. O meio foi substituído e os estímulos reaplicados a cada 48 horas durante os 6 dias de experimento. (Figura adicional 3A): Resultado quantitativo do número de osteoclastos por poço. (Figura adicional 3B): Imagens representativas obtidas no microscópio de fluorescência, de cada condição experimental (azul: núcleos celulares marcados com DAPI, verde: actina citoplasmática marcada com faloidina conjugada com AlexaFluor488. As barras indicam os valores médios e as linhas verticais indicam o erro padrão da média (SEM) de três experimentos diferentes avaliados em triplicado. * p<0,001 em relação ao grupo controle sem ligadura Ø+, # p<0,001 em relação ao grupo controle + RankL. ANOVA seguida de teste post-hoc de Tukey.

Fonte: Elaboração própria.

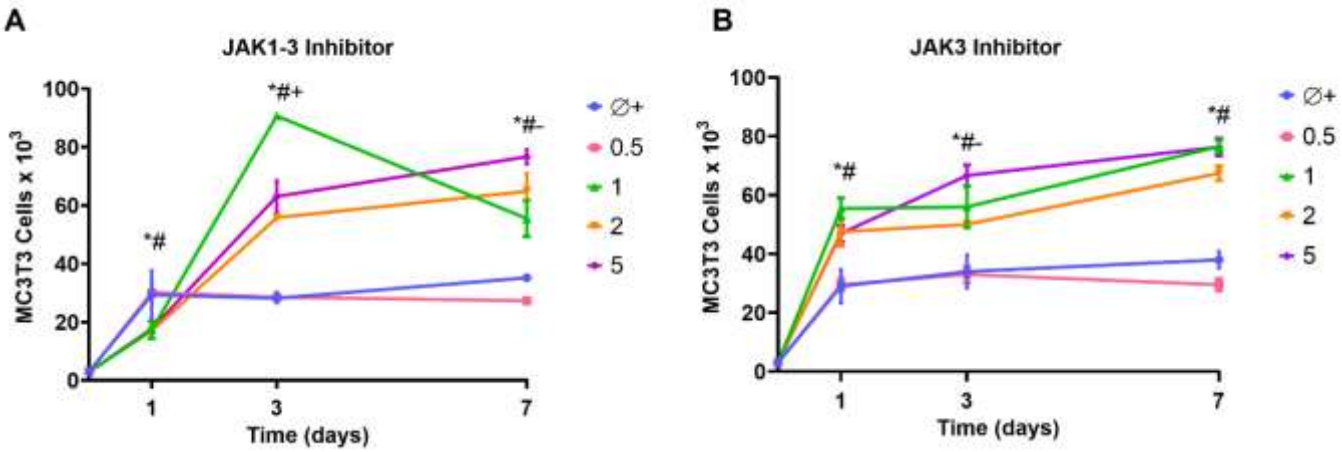
Figura adicional 4 - Inibidores de JAK reduzem significativamente a produção de ROS induzida por LPS em macrófagos (RAW 264.7).



As barras indicam a porcentagem média de ROS produzidos por macrófagos após pré-tratamento dos inibidores de JAK1-3 e JAK3, e as linhas verticais o erro padrão da média (SEM). Enquanto JAK1-3 inibiu de maneira dose-dependente a produção de ROS, todas as concentrações de JAK3 foram capazes de inibir a produção de ROS induzida pelo LPS (A). Estratégia de *gating* para imunofluorescência de macrófagos (RAW 264.7): A utilização do dotplot que cruza o tamanho celular (FSC) com a complexidade celular (SSC) permitiu a identificação da população de interesse (P1=63%) (B). Considerando apenas a população de interesse (gate P1), foram excluídos os *doublets* e mantidos os *singlets* (P2=95,77%) (C). Utilizando a porta "P2" foi identificada a produção de ROS+ (40,73%) (D). * p<0,05 em relação ao grupo controle (Ø), estimulado apenas com LPS. ANOVA seguida de teste pos-hoc de Tukey.

Fonte: Elaboração própria.

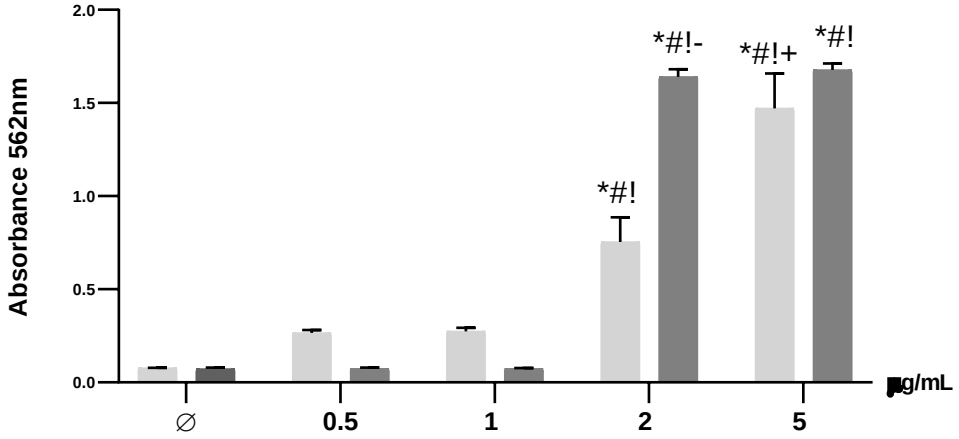
Figura adicional 5 - Inibidores de JAK aumentam a proliferação de células pré-osteoblásticas (MC3T3-E1).



As células foram plaqueadas com $3x10^3$ células por poço e cultivadas com α-MEM suplementado com concentrações de inibidor de JAK1-3 e JAK3, por 1, 3 e 7 dias. Após os períodos, as células foram contadas com auxílio de azul de tripan para avaliar o aumento ou diminuição da proliferação celular. As maiores concentrações (1, 2 e 5 μg/ml, de ambos inibidores, aumentaram significativamente a proliferação celular nos períodos mais tardios – 3 e 7 dias. (A) Resultado quantitativo do número de células pré-osteoblásticas por poço utilizando inibidor JAK1-3. (B) Resultado quantitativo do número de células MC3T3 por poço utilizando o inibidor JAK3. As barras indicam os valores médios; linhas verticais indicam o erro padrão da média de três experimentos diferentes avaliados em triplicata. As colunas representam a média ± SEM. *p<0,05 mostra a diferença dos grupos 1, 2 e 5 µg/mL em relação ao Grupo Controle (Ø) em cada período, #p<0,05 mostra a diferença entre os grupos 1, 2 e 5 µg/mL em relação ao grupo 0,5 µg/mL em cada período ao utilizar ambos inibidores, +p<0,05 mostra a diferença, no referente período, entre os grupos 2 e 5 µg/mL em relação ao grupo 1 µg/mL nos experimentos com JAK1-3i, -p<0,05 mostra a diferença, no referente período, entre os grupos 1 e 2 µg/mL em relação ao grupo 5 µg/mL, utilizando ambos inibidores. ANOVA seguida de teste post-hoc de Tukey.

Fonte: Elaboração própria.

Figura adicional 6 - O tratamento com os inibidores de JAK em células pré-osteobásticas estimulam a formação de nódulos de mineralização.



Células MC3T3-E1 foram plaqueadas com $3x10^3$ células por poço e cultivadas com α-MEM suplementado com concentrações de inibidor de JAK1-3 e JAK3, por 14 dias. Após os períodos as células foram fixadas, lavadas e em seguida foi adicionada solução de vermelho de alizarina. Após a retirada do corante, foi adicionado cloreto de cetilpiridínio para solubilizar os nódulos de cálcio corados com vermelho de alizarina para leitura em espectrofotômetro. As maiores concentrações (2 e 5µg/ml) de ambos os inibidores, foram capazes de estimular maior formação de nódulo de mineralização no período de 14 dias. Interessantemente, a concentração de 0,5µg/ml do inibidor de JAK3 também provocou aumento da mineralização. As barras indicam os valores médios; linhas verticais indicam o erro padrão da média de três experimentos diferentes avaliados em triplicata. As colunas representam a média. *p<0,001 mostra a diferença em relação ao Grupo Controle (Ø), #p<0,001 mostra a diferença em relação ao grupo tratado com o mesmo inibidor na concentração de 0,5 µg/mL, !p<0,001 mostra a diferença com o grupo tratado com o mesmo inibidor na concentração de 1 µg/mL, + p<0,001 mostra a diferença com o grupo tratado com o mesmo inibidor na concentração de 2 µg/mL e - p< 0,001 mostra a diferença entre os grupos na mesma concentração de 2 µg/mL. ANOVA seguida de teste post-hoc de Tukey.

Fonte: Elaboração própria.

ANEXO A - APROVAÇÃO DO COMITÊ DE ÉTICA (CEUA)

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

FACULDADE DE ODONTOLOGIA

CERTIFICADO

Certificamos que a proposta intitulada *"EFEITO DA INIBIÇÃO DE JAK SOBRE A PROGRESSÃO DA DOENÇA PERIODONTAL. ESTUDO IN VIVO E IN VITRO"* registrada com o nº **27/2020**, sob a responsabilidade da **Prof(a). Dr(a). Morgana Rodrigues Guimarães Stabili** – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA FACULDADE DE ODONTOLOGIA DE ARARAQUARA** em reunião de 25/11/2020.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	Janeiro/2024
Espécie/linhagem/raça	Rato / Rattus norvergivus albinus/Holtzman
Nº de animais	96
Peso/Idade	100 a 200 gramas/08 semanas
Sexo	Macho
Origem	Biotério Central da FOAr (Câmpus-Araraquara)

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

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Araraquara, 15 de março de 2024.

Mariely Araújo de Godoi