Universidade Estadual de Londrina

ANA CARLA ZARPELON

AVALIAÇÃO DO EFEITO TERAPÊUTICO E MECANISMO DE AÇÃO DA BUDLEÍNA A EM MODELOS DE ARTRITE EM CAMUNDONGOS.

Londrina
2011

ANA CARLA ZARPELON

AVALIAÇÃO DO EFEITO TERAPÊUTICO E MECANISMO DE AÇÃO DA BUDLEÍNA A EM MODELOS DE ARTRITE EM CAMUNDONGOS.

Dissertação apresentada à Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina para obtenção do Título de Mestre em Patologia Experimental.

Orientador: Waldiceu Aparecido Verri Jr.

Londrina
2011

Catalogação elaborada pela Divisão de Processos Técnicos da Biblioteca Central da Universidade Estadual de Londrina.

Dados Internacionais de Catalogação-na-Publicação (CIP)

Z38a Zarpelon, Ana Carla.
Avaliação do efeito terapêutico e mecanismo de ação da budleína a em modelos de artrite em camundongos. / Ana Carla Zarpelon. – Londrina : 2011.

103 f. : il.

Orientador:Waldiceu Aparecido Verri Junior.
Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Centro de Ciências Biológicas, 2011.
Inclui bibliografia.

1. Budleína– Teses. 2. Hipernocicepção – Teses. 3. Artitre– Teses 4. Gota – Teses I. Verri Junior, WIaldiceu Aparecido. II. Universidade Estadual de Londrina. III. Título.

CDU 676.72

ANA CARLA ZARPELON

AVALIAÇÃO DO EFEITO TERAPÊUTICO E MECANISMO DE AÇÃO DA BUDLEÍNA A EM MODELOS DE ARTRITE EM CAMUNDONGOS.

Dissertação apresentada à Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina para obtenção do Título de Mestre em Patologia Experimental.

BANCA EXAMINADORA

_____

Prof. Dr. Thiago Mattar Cunha
Universidade de São Paulo – USP

_____

Prof. Dr. Wander Rogério Pavanelli
Universidade Estadual de Londrina – UEL

_____

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior
Universidade Estadual de Londrina – UEL

Londrina, 25 de Fevereiro de 2011.

Trabalho realizado no laboratório de Dor, Inflamação, Neuropatia e Câncer do Departamento de Ciências Patológicas, no Centro de Ciências Biológicas da Universidade Estadual de Londrina, com o auxílio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Dedicatória

À aqueles que não são apenas pais, são amigos, parceiros, irmãos, confidentes. A admiração é a única forma que encontro para defini-los, obrigada por me apoiarem em todos os momentos. Ao meu amor, sempre comigo nessa caminhada, acreditando nos meus sonhos e fazendo deles os seus. E a família que me acolheu como filha e irmã, se isso foi possível, vocês também foram os grandes condutores.

Agradecimentos

Ao professor **Dr. Waldiceu Ap. Verri Jr.**, pela orientação, apoio e imensa confiança em meu trabalho. Agradeço pelas oportunidades de trabalhar em seu laboratório e também por não medir esforços para meu aprendizado.

Aos professores **Thiago Mattar Cunha** e **Wander Rogério Pavanelli** pela disponibilidade de participar na minha banca de qualificação, engrandecendo as discussões e possibilitando sugestões.

Aos professores **Sérgio Henrique Ferreira, Fernando Queiróz Cunha** e **Thiago Mattar Cunha** pela oportunidade de realizar diversos experimentos em seus laboratórios, sendo muito importante para meu amadurecimento científico.

Aos docentes do departamento de Patologia experimental, **Phileno Pinge Filho, Wander R. Pavanelli, Maria Angélica Watanabe, Rodrigo Luiz, Rubens Cecchini, Mário Augusto Ono, Eiko Nakagawa Itano**, **Emerson Venâncio, Halha Saridakis, Marli Pinge,** pela contribuição para com a minha formação científica.

Aos meus amigos **Larissa, Fabrício, Walter, Jhimmy, Rafael e Guilherme** pela dedicação e competência que foram de fundamental importância para a realização deste trabalho, e também pela confiança e incentivo. Muito obrigada por estarem presentes nas discussões, e experimentos que foram decisivos para o engrandecimento deste.

A todos os amigos e colegas do Laboratório de Dor, Inflamação Neuropatia e Câncer da Universidade Estadual de Londrina, **Ana Clara, Bárbara, Carla, Cássia, Felipe, Gabriela, Giovana, Larissa, Mab, Miriam, Paula, Renata, Renato, Sandra, Sérgio, Thacyana, Victor** pela agradável convivência e imenso bom humor.

Aos amigos da turma do **Mestrado em Patologia Experimental de 2009**, pelo convívio e aprendizado. Muito obrigado e sucesso a todos.

Aos meus pais, **Zarpelon e Eliana**, pela paciência e orgulho, que fazem os meus dias parecerem mais fáceis. Se hoje sou alguém isso é graças a vocês, que estiveram presentes na formação do meu caráter. Obrigada pelo incentivo, suporte, e por estarem sempre ao meu lado, mesmo que ás vezes isso foi muito custoso.

Ao **João Luiz**, uma pessoa muito especial que esteve sempre me apoiando, me incentivando e contando como se tudo já estivesse certo, mesmo sem ter acontecido. Obrigado meu amor, por estar ao meu lado! E a família **Schütz**, que também nunca mediu esforços para me apoiar, seja com palavras de incentivo, seja com férias inesperadas, obrigado por me incluírem em seu convívio familiar.

Á minha prima **Rúbia**, a irmã que a vida me apresentou recentemente, por seus conselhos valiosos, e por todo aprendizado que inseriu em mim. Obrigado por fazer de mim uma pessoa melhor, meu eterno carinho, admiração e respeito.

A família **Casagrande**, pela paciência, empenho, carinho. Obrigado por não medirem esforços fazendo do seu lar o meu, muito obrigada pelo carinho com que fui recebida, contem comigo sempre!

A minha grande amiga querida **Francielle**, que soube como me incentivar nos momentos que realmente precisei. Que me impulsiona sempre!

Ao apoio técnico, **Jesus, Pedro, Ieda e Sérgio, Kátia, Diva, Fabíola**, pelo convívio e amizade.

A todas as pessoas, que provavelmente, por um momento de descuido, acabei por não mencionar os nomes, mas que contribuíram direta ou indiretamente, mas não de maneira menos importante para a realização desde trabalho, deixo o meu muito obrigada.

“Nem tudo que se enfrenta pode ser modificado, mas nada pode ser modificado até que seja enfrentado”.

Albert Einsten

Resumo

ZARPELON, Ana Carla. **Avaliação do efeito terapêutico da budleína A em modelo de artrite em camundongos.** 2011. 103f. Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2011.

RESUMO

A artrite reumatóide é uma doença crônica, auto-imune, caracterizada por lesões articulares graves, recrutamento de linfócitos tanto Th1 quanto Th17, e produção das citocinas envolvidas nesses padrões de resposta. A gota também é um tipo de artrite, porém esta é caracterizada pela deposição de cristais de urato monossódico nas articulações e tecidos periarticulares, apresentando dor e rigidez intensa. Existe atualmente uma série de terapias, como por exemplo, as que inibem a ação das citocinas, anti-TNF e anti-IL-1, e os glicocorticóides que de maneira receptor-dependente inibem a ativação e/ou atividade do fator de transcrição NFκB. Contudo, apesar da inibição da atividade/ativação do NFκB ser um mecanismo antiinflamatório eficaz, os efeitos adversos relacionados à atividade hormonal dos glicocorticóides limitam o seu uso. Dessa forma, a inibição da ativação do NFκB por drogas que não apresentem efeitos adversos hormonais semelhantes aos glicocorticóides é uma estratégia promissora. Nesse sentido, foi demonstrada recentemente a atividade da budleína A, uma lactona sesquiterpênica, em um modelo de inflamação inata aguda induzida por carragenina, o qual está relacionado à inibição da produção de citocinas como TNFα e IL-1β. Outro estudo demonstrou in vitro que a budleína A inibe a ativação do NFκB. Porém, não foi investigada a sua atividade em modelos de artrite. Assim, propomos no presente estudo a investigação do efeito terapêutico da budleína A em modelo de artrite induzida por antígeno e gota induzida pela administração intra-articular de cristais de urato monosódico. O modelo de artrite induzida pela imunização e desafio intra-articular com antígeno protéico é um modelo de resposta auto-imune com ativação do fator transcricional pró-inflamatório NFκB, bem como o modelo de gota em camundongos. Neste trabalho, pudemos observar que o tratamento com a budleína A inibiu a inflamação (hiperalgesia mecânica, edema e recrutamento de leucócitos), e a produção de citocinas características nos modelos de artrite induzida por antígeno e gota. Dessa forma, os resultados demonstram a aplicabilidade da budleína na artrite reumatóide e gotosa por inibir o processo inflamatório relacionado à produção de citocinas provavelmente por um mecanismos dependente da inibição da ativação do NFkB.

Palavras-chave: Budleína. Hipernocicepção. Artrite. Gota. Dor inflamatória. Citocinas. Migração de neutrófilos.

Abstract

ZARPELON, Ana Carla. **Evaluation of the therapeutic effect of budlein A on models of arthritis in mice.** 2011. 103f. Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2011.

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by articular lesions, recruitment of Th1 and Th17 lymphocytes, and production of cytokines related to these lymphocytes. Moreover, the gout is also a type of arthritis, but is characterized by accumulation of monosodium urate crystals in joints and periarticular tissues resulting in intense inflammation with pain and stiffness. In this sense, cytokine targeting therapies such as anti-TNF and anti-IL-1, and glucocorticoids that inhibit the activation of the transcription factor NFκB acting on glucocorticoid receptors are effective strategies in arthritis. Nevertheless, although inhibition of NFκB activity is an effective anti-inflammatory mechanism of glucocorticoids, their adverse hormonal effects limit their use. Therefore, a drug that inhibits NFκB activation without the adverse side effects of glucocorticods would be a suitable therapeutic strategy. Recent studies demonstrated that the sesquiterpenic lactone, budlein A, inhibits innate inflammation induced by carrageenin, which is associated to cytokines production (TNFα and IL-1β) inhibition. Another study demonstrated in vitro that Budlein A inhibited NFκB activation. However, it is not known whether budlein A represents a possible therapeutic approach in diseases related to NFκB activation such as rheumatoid arthritis and gout arthritis. Therefore, in the present study we evaluated the therapeutic effect of budein A in antigen induced-arthritis (AIA) and gouty arthritis induced by mono-sodium urate crystals injection directly on the joint. The antigen induced-arthritis and gout arthritis models course are dependent on NFκB-dependent cytokine production. Herein, it was detected reduced inflammation (mechanical hyperalgesia, odema and leucocytes recruitment) and cytokine production in the antigen-induced arthritis model in a 6 day schedule budlein A treatment with no detectable liver side effects. Similar results were obtained in the gout arthritis model. These results demonstrate that budlein A treatment inhibits prolonged inflammation in antigen-induced and gouty arthritis by inhibiting the inflammatory process related to cytokine production, likely to be dependent on the NFκB activation.

Keywords: Budlein A. Hypernociception. Arthritis. Gouty. Inflammatory pain. Cytokines. Neutrophil migration.

Lista de abreviaturas

LISTA DE SIGLAS E ABREVIATURAS

AINES	Antiinflamatórios não esteroidais
AMPC	Adenosina monofosfato cíclico
AR	Artrite reumatóide
CFA	Adjuvante completo de Freund's
COX	Cicloxigenase
CXCL1	Quimiocina ligante 1
ET-1	Endotelina-1
g	Gramas
i.a	Via intraarticular
IL	Interleucina
IL-1β	Interleucina-1 beta
KC/CXCL1	Quimiocina derivada de queratinócitos
LTB4	Leucotrieno B4
mBSA	Albumina sérica bovina metilada
MPO	Mieloperoxidase
MSU	Urato monossádico
PGE2	Prostaglandina E2
PKA	Proteína quinase A
p.o.	Via oral
s.c.	Via subcutânea
STLs	Lactonas sesquiterpênicas
TLR	Receptores semelhantes ao Toll
TNFα	Fator de necrose tumoral alfa
Th1	Linfócito T auxiliary CD4+1
Th2	Linfócito T auxiliary CD4+2
Th17	Linhagem celular Th17
V. robusta	Viguiera robusta

Sumário

SUMÁRIO

1. Introdução

1.1. Inflamação

A capacidade do organismo em desenvolver uma resposta inflamatória é fundamental a sobrevivência e manutenção da homeostasia em vista aos patógenos e lesões ambientais à que estamos susceptíveis. A inflamação é geralmente acompanhada de sinais cardinais: edema, calor, rubor, dor e perda da função (Insel et al., 1996; Gallyn & Snyderman, 1999).

As respostas inflamatórias ocorrem de modo geral em três fases diferentes, cada qual aparentemente mediada por mecanismos diversos: 1. fase transitória aguda caracterizada por vasodilatação de arteríolas, capilares e vênulas, e com aumento da permeabilidade, do fluxo sanguíneo e exsudação de proteínas do plasma; 2. fase subaguda ou tardia, marcada principalmente pela infiltração dos leucócitos e células fagocitárias; e 3. fase proliferativa crônica, na qual há degeneração tecidual e fibrose (Insel et al., 1996; Gallin & Snyderman, 1999).

O processo inflamatório é essencial para sobrevivência de qualquer indivíduo, porém este quando exacerbado causa desequilíbrio na homeostase. Anteriormente, acredita-se que as características da inflamação constituíam processos independentes, tais como dor, edema e migração leucocitária. Porém, Wedmore e Williams (1991) demonstraram que diferentes agentes quimiotáticos podem desencadear a migração de leucócitos, e que estes têm participação central no aumento da permeabilidade e formação do exsudato, bem como no desencadeamento da hiperalgesia inflamatória (Levine et al., 1984; Bisgaard & Kristensen, 1985).

Devido à vasodilatação de arteríolas, capilares e vênulas, advém o aumento da permeabilidade vascular, culminando em hiperemia vascular e, conseqüentemente, aumento da temperatura local bem como o rubor ou vermelhidão.

Um importante componente da resposta inflamatória é o recrutamento de leucócitos (ex. neutrófilos, monócitos e linfócitos) para o foco inflamatório (Valerio et al., 2007; Verri et al., 2007 e 2010; Souto et al., 2011). Num primeiro momento, neutrófilos são recrutados por citocinas (fator de necrose tumoral e interleucina -1β) levando a fagocitose e eliminação do agente infeccioso no foco inflamatório. Todavia, a inflamação excessiva causa dano ao tecido (Lemos et al., 2009; Verri et al., 2010).

A dor, por sua vez, é originada de mecanismos mais complexos, sendo definida pela Associação Internacional para o Estudo da Dor (IASP) como “uma experiência sensorial e emocional desagradável associada ou não a uma lesão tecidual” (Flórez et al., 1993). Para compreender os mecanismos associados à dor, é necessário o esclarecimento de alguns termos. A nocicepção (do latim *nocere*, “ferir”), é o resultado da detecção de estímulos capazes de comprometer a integridade física de um organismo (estímulos nociceptivos). Além disso, existem receptores sensoriais específicos os quais detectam a percepção de estímulos prejudiciais pelo sistema nervoso central, chamados de nociceptores (Basbaum and Jessell, 2000). A estimulação dos nociceptores periféricos faz com que a informação nociceptiva seja conduzida através das fibras aferentes primárias (neurônios de primeira a ordem) ao sistema nervoso central (SNC). Além disso, outros termos são utilizados na prática experimental relacionados a dor inflamatória, tais como alodinia (dor decorrente de um

estímulo normalmente não doloroso) e hiperalgesia (resposta exacerbada a um estímulo doloroso).

As respostas nociceptivas são conduzidas através de fibras, as quais se dividem em duas classes maiores (Meyer et al, 2008). A primeira inclui as fibras C, finas (diâmetro de 0,4 a 1,2 mm), não mielinizadas, e de condução lenta (0,5 a 2 m/s) responsáveis pela dor de longa duração, com localização pobre e difusa, estas são conhecidas como polimodais, pois são capazes de responder a estímulos mecânicos, térmicos e quimicos. A segunda classe são as fibras Aδ, sendo essas de diâmetro médio (2 a 6 mm), pouco mielinizadas, de condução intermediária (2 a 30 m/s), responsáveis pela dor de curta duração (dor aguda) (Meyer et al, 2008). Além das fibras Aδ, seus subtipos e as fibras C, existe outro tipo de fibras, conhecidas como Aβ, as quais são amplamente mielinizadas, e de grande diâmetro, responsáveis por estímulos inócuos aplicados a pele, músculos (consideradas táteis), e normalmente não contribuem para a dor. Porém, podem passar a responder como nociceptores em certas patologias, como na neuropatia onde ocorre uma plasticidade neuronal (Bausbam, 2009). Outra população de nociceptores são os silenciosos ou “sleeping”, que tornam se responsivos somente quando sensibilizados (Jullius and Basbaum, 2001).

Quanto à hiperalgesia mecânica na inflamação por estímulo como a carragenina, sendo este um modelo clássico para estudo da fisiopatologia da dor, existem evidências da participação de mediadores indiretos e diretos. Os mediadores indiretos são constituídos pelas citocinas pró-inflamatórias como o TNFα, IL-1β e quimiocina CXCL1 em camundongos (IL-8 em humanos e CINC-1 em ratos). Essas citocinas agem de maneira coordenada e interdependente

no sentido de que após o estímulo como a carragenina, ocorre a produção de TNFα e CXCL1. Ambas induzem a produção de IL-1β que por sua vez ativa a ciclooxigenase (COX) e ocorre a produção de prostaglandina E_2 (PGE_2). A CXCL1 também induz a liberação de aminas simpáticas. Essa cascata de citocinas representa os mediadores indiretos, pois, atuam via liberação de mediadores diretos como a PGE_2 e aminas simpáticas que atuam diretamente em seus receptores nos neurônios nociceptivos aferentes primários causando a sensibilização desses neurônios (Cunha et al., 2005; Verri et al., 2006).

A sensibilização induzida pelos mediadores diretos como a PGE_2 se refere a dois fenômenos: 1) a PGE_2 sensibiliza nociceptores que estão normalmente silenciosos (“sleeping nociceptors”) durante o processo inflamatório, e como resultado há maior número de fibras nociceptivas sendo ativadas durante a inflamação; 2) a PGE_2 facilita a despolarização induzida por outros mediadores químicos como a bradicina, estímulos térmicos ou mecânicos de alta intensidade. Essa facilitação da despolarização neuronal resulta da ativação dos receptores para PGE_2 acoplados à proteína Gs, que estimulam a adenilato ciclase com conseqüente ativação da proteína quinase dependente do AMPc (PKA), a qual fosforila canais de sódio sensíveis a tetrodotoxina causando a sensibilização neuronal. Contudo, o aumento intra-neuronal de sódio induzido pela PGE_2 em doses relacionadas com a hiperalgesia mecânica não é suficiente para despolarização do neurônio, mas facilita a despolarização induzida por outros mediadores e estímulos. Dessa forma, os mediadores diretos ou finais causam a sensibilização dos neurônios nociceptivos e sua produção está intimamente relacionada com a produção prévia de citocinas pró-inflamatórias (Verri et al., 2006).

Além das citocinas pró-inflamatórias existe a participação de outras moléculas pró-nociceptivas como o peptídeo endotelina-1 (ET-1). A ET-1 medeia a hiperalgesia induzida por citocinas como TNFα, IL-1β, IL-12, IL-15, IL-18 e IL-33, e a hiperalgesia induzida pela ET-1 depende pelo menos parcialmente da produção de PGE_2 (Conte et al.,2008; Verri et al., 2008; Khodorova et al., 2004).

Por outro lado, citocinas como a IL-10 tem um papel antinociceptivo endógeno porque reduzem a produção de TNFα e IL-1β. Dessa forma, a inibição da atividade da IL-10 resulta em aumento da hiperalgesia induzida pela carragenina e em outros modelos inflamatórios também ocorre à exacerbação do processo. Ademais, o tratamento com IL-10 reduz a hiperalgesia no modelo da carragenina e em modelos de dor neuropática (Poole et al., 1995; Milligan et al., 2005; Milligan et al., 2006).

Assim, verifica-se que a habilidade de detectar estímulos potencialmente nociceptivos pelo sistema somatosensorial é um mecanismo de proteção muito importante que envolve a interação de vários mecanismos periféricos e centrais (Cousins & Cohen, 2005). A perda de função resultante do processo inflamatório pode ser exemplificada na artrite reumatóide, na qual o edema articular e a dor limitam a movimentação do membro ao passo que a lesão da cartilagem e mesmo tecido ósseo resultam da ativação de células residentes e migratórias que reconhecem estruturas próprias como sendo antígenos e produzem diversas proteases (McInnes & Liew, 2005).

1.2. Artrite Reumatóide

A artrite reumatóide (AR) é uma doença inflamatória crônica auto-imune de etiologia desconhecida, apresentando manifestações locais e sistêmicas, tais como aumento de infiltrado de células imunes (neutrófilos, células T, B e macrófagos) no interior da membrana sinovial, cavidades e tecidos periarticulares (Pinto et al., 2009; Harris et al., 1990; Firestein et al., 2003). As causas atualmente descritas como prováveis desencadeadoras da AR são, a presença de um artritogênico exógeno primário (agente infeccioso inespecífico), susceptibilidade genética, uma reação auto-imune tendo como alvo as membranas sinoviais e mediadores inflamatórios promovendo o dano articular (Harris et al., 1990).

A AR é uma inflamação poli-articular simétrica que acomete principalmente e primeiramente em pequenas articulações das mãos, pés até joelhos e cotovelos. Esta é capaz de gerar uma alteração irreversível da cápsula e da cartilagem articular, já que além da inflamação da membrana sinovial ocorre a formação do pannus (interface entre a cartilagem e o sítio de erosão ativa) aumentando a rigidez, impossibilitando assim a movimentação adequada.

Quanto à sensibilização periférica durante a inflamação das articulações, numerosos neurônios aferentes primários são sensibilizados pelos mediadores inflamatórios produzidos. Assim, estímulos sublimiares antes não nociceptivos para os mecanoreceptores acarretam em uma resposta exacerbada frente a estímulos mecânicos como os que ocorrem na movimentação. Ou seja, os nociceptores tornam-se sensibilizados e preparados para responder a baixas

pressões articulares e a qualquer movimento que necessite de alguma atividade das articulações. Várias unidades das fibras mielinizadas finas (Aδ) ou não mielinizadas (fibras C) e também os nociceptores silenciosos tornam-se responsivos a estimulação mecânica nas articulações e contribuem para ativação dos neurônios aferentes primários durante a inflamação (Schaible et al., 2002).

Outro fator relevante na AR é a presença de inúmeras células nos fluidos e cavidade articular, e peri-articulares incluindo um grande número de neutrófilos, células T, células B e macrófagos, além da proliferação de sinoviócitos do tipo fibroblastos e do tipo macrófago na cavidade articular e sinóvia tornam a camada de revestimento hiperplásica. Além disso, começam a ser expressas no local enzimas de degradação, incluindo metaloproteinases, serina proteases e agrecanases, as quais digerem-se a matriz extracelular ocasionando erosão e destruição da cartilagem e osso (Firestein et al., 2003; Harris et al., 1990).

Com a intensa participação celular na AR, o infiltrado inflamatório tem sido associado aos principais danos teciduais nesta doença. Os neutrófilos são a maioria, chegando a somar 90% do infiltrado leucocitário no fluido sinovial, na cartilagem e no pannus dos pacientes acometidos pela AR (Hollingsworth et al., 1967; Mohr et al., 1995). O neutrófilo permanece viável no foco inflamatório cerca de 24-48 hs após o seu recrutamento, entrando em apoptose e sendo progressivamente substituído por mononucleares. Em doenças crônicas como a AR, o neutrófilo pode permanecer por mais tempo na articulação, sendo responsável em grande parte pela lesão articular. De fato, a supressão do gene

FOXO3a, o qual reprime a expressão de *fas* ligante, induz a apoptose de neutrófilos reduzindo a lesão articular em modelo de AR (Jonsson et al., 2005).

Os neutrófilos são células secretoras e pela interação com uma ampla variedade de estímulos (mediadores produzidos pelas células residentes ou componentes do próprio antígeno) no foco inflamatório liberam agentes microbicidas, pró-inflamatórios e que degradam a matriz extracelular. Pelo menos três vias diferentes foram descritas em fagócitos. Dentro de segundos após a interação dos neutrófilos com agonistas, ocorre à liberação de espécies reativas do oxigênio e produtos lipídicos. Logo depois, dentro de segundos-minutos, ocorre à liberação de enzimas pré-formadas e proteínas que estavam armazenadas dentro de vesículas. Finalmente, a ativação da transcrição de genes resulta (dentro de poucas horas) na produção e secreção de citocinas pró-inflamatórias (Berton et al., 1999). Os neutrófilos expostos a uma variedade de citocinas pró-inflamatórias aumentam sua atividade na liberação dos mediadores citotóxicos já citados e também das próprias citocinas, aumentando assim a destruição articular (Feldmann et al., 1990).

Keystone em 1977 demonstrou pela primeira vez que a administração intraarticular de zymosan (componente da parede celular de fungos), apresentava características semelhantes a AR, com degradação articular, formação de pannus, infiltrado neutrofílico e hipertrofia. Após algum tempo, Guerreiro et al. 2006, descreveu um modelo para avaliação da hipernocicepção articular induzida por zimosan que consiste na flexão da articulação inflamada.

Existem alguns modelos para o estudo da inflamação que se assemelham a artrite reumatóide. Alguns utilizam a administração de

componentes microbianos diretamente na articulação, outros utilizam a imunização dos animais com desafio local ou sistêmico com o antígeno.

Um exemplo de modelo com características da artrite reumatóide é o de artrite induzida pela imunização e desafio com metil éster da albumina do soro bovino (mBSA). Neste protocolo é utilizada como adjuvante o CFA, que induz uma polarização da resposta inflamatória para Th1/Th17. Neste modelo já foram demonstradas as lesões articulares e participação de diversas citocinas, bem como o recrutamento de células como os neutrófilos, edema e hiperalgesia mecânica (Brackertz et al., 1977).

A administração do antígeno (mBSA) intraplantar ou articular tíbio-tarsal de camundongos previamente imunizados produz diminuição do limiar nociceptivo de forma dose e tempo dependente, sendo o efeito hipernociceptivo intraplantar dependente da liberação de TNF-α, IL-1β e KC, via liberação de prostanóides e aminas simpáticas (Cunha et al., 2008; Verri et al., 2008). Verri et al. (2008) demonstrou a participação da IL-33 (uma citocina pró-inflamatória da família da IL-1) na hipernocicepção articular e cutânea induzida por antígeno, antecedendo a liberação dos mediadores descritos acima.

A dor é uma das principais queixas do paciente com artrite reumatóide, uma vez que o impossibilita das atividades diárias, comprometendo a qualidade de vida do indivíduo. As terapias atuais para o controle da artrite reumatóide apresentam efeitos colaterais como a imunossupressão o que se confirma pelo aumenta da incidência de artrite séptica em pacientes com artrite reumatóide (corticosteróides, imunobiológicos) (Favero et al., 2008; Ma et al., 2009) ou o aumento do risco de incidentes cardiovasculares pela inibição da COX-2

endotelial (inibidores altamente seletivos para a COX-2). Nesse sentido, faz-se necessária a busca de novas terapias. Não é necessário que as novas terapias não apresentem efeitos colaterais ou mesmo não apresentem outros efeitos colaterais, mas novas possibilidades terapêuticas possibilitarão a escolha da melhor abordagem terapêutica para cada paciente com AR.

1.3. Gota

A gota é uma forma de artrite causada pela deposição de cristais de urato monossódico (MSU) nas articulações e tecidos adjacentes. Os cristais de MSU induzem uma reação inflamatória que se manifesta com intensa dor, edema e eritema (Chia et al., 2008; Schweyer et al., 2000). Ademais, apesar de não ser um sinal clínico da gota, esta também é caracterizada pelo e infiltrado neutrofílico intenso. Estes neutrófilos apresentam papel importante como produtores de ânion superóxido e radicais livres, citocinas pró-inflamatórias e metaloproteases (Chia et al., 2008).

A artrite gotosa é conseqüência de desordens metabólicas comuns que gerem a hiperuricemia crônica, que por sua vez resulta na deposição crônica de cristais de MSU nos tecidos em aglomerados microscópicos e as vezes macroscópicos.

A gota pode se classificar como primária e secundária. A forma primária é de causa desconhecida e tem algum componente genético (hereditário), sendo a mais comum. Já a secundária desenvolve-se em consequência de outra doença ou alguns medicamentos. Dentre as doenças que estão associadas a gota pode-se citar: doenças hemolíticas, doenças mieloproliferativas, insuficiência renal, obesidade, hipertensão arterial,

hipotireoidismo, etc. A ingestão de bebida alcoólica é também uma causa comum de hiperuricemia, podendo causar a gota.

Associados às deficiências/alterações metabólica, existem quadros de agudização, nos quais, cerca de 1% da população adulta é alvo de eventos específicos como trauma, cirurgia, excesso de álcool ou drogas, que podem afetar os níveis de urato no soro. Tais eventos podem estimular a formação dos cristais de MSU, os quais precipitam nos tecidos, tornando os fluidos supersaturados (Tak et al., 1980).

Atualmente o que se tem é que a liberação de citocinas parece ser um evento importante na resposta inflamatória e conseqüente patofisiologia da gota. Em particular, citocinas como a interleucina-1 (IL-1β), TNF-α e IL-8 de monócitos e macrófagos que tentam realizar a fagocitose dos cristais de urato tem papel central na iniciação e propagação da resposta inflamatória.

Nesta doença reumática as primeiras células a serem recrutadas são os neutrófilos, os quais se acumulam nos fluidos articulares e na membrana sinovial sofrendo membranólise, geração de radicais livres, liberação de enzimas lisossomais, prostagladina E_2, leucotrienos e interleucinas como a IL-1β (Terkeltaub et al, 2004). Acredita-se então que estes eventos são importantes para a inflamação decorrente dos cristais de urato (Rasool et al., 2006).

No organismo, os cristais de MSU podem apresentar em sua surperfície inúmeros ligantes reativos dentre mais de 25 proteínas séricas, e também proteínas de membrana como as integrinas (Terkeltaub et al., 1983; Jaques et al., 1982; Barabe et al., 1998). Assim, os cristais de MSU são capazes de ativar tanto a via clássica quanto a via alternativa do sistema complemento.

Outro grupo de receptores que participam da fisiopatologia da gota são os receptores semelhantes ao Toll (TLR). Segundo Liu-Bryan em 2005, a estrutura e função dos TLR-2 e TLR-4 contribuem para variabilidade no fenótipo clínico da gota em humanos. Nesse estudo, eles demonstram que tanto os TLR-2 e TLR-4 e sua proteína acessória MyD88 participam da inflamação em um ambiente livre de endotoxinas.

O tratamento da gota se divide em dois períodos: o tratamento das crises e a profilaxia destas. No momento das crises são utilizados antiinflamatórios não esteroidais (AINEs) e colchicina. A colchicina é menos tóxica do que os anti-inflamatórios (especialmente para os rins e estômago) e controla a gota eficazmente, mas pode causar efeitos colaterais desagradáveis, como náuseas, vômitos e diarréia. Uma vez cessada a crise de gota, o tratamento se volta para a diminuição dos níveis de ácido úrico, e a droga mais usada para este objetivo é o alopurinol, tendo como efeitos colaterais as reações de hipersensibilidade. Conhecendo os efeitos colaterais fica clara a necessidade do desenvolvimento de novas terapias (Martin et al, 2009).

1.4. Budleína A

A atividade medicinal de algumas plantas, incluindo a família Asteraceae, é atribuída ao seu conteúdo de lactônas sesquiterpênicas incluindo micanolido, helenalina, partenolido, artemisinina, glutarato de bis (isoalantodiol-B). As atividades *in vitro* das lactonas sesquiterpênicas englobam efeitos antimicrobianos (Pickman et al., 1986), antivirais (Meshnick et al., 2002) e antitumorais (Chen et al., 1994).

As lactonas sesquiterpênicas também são drogas antiinflamatórias promissoras. Elas inibem a formação de edema em modelos de granuloma por algodão, adjuvante completo de Freund, 4-beta-forbol 12-miristato 13-acetato, formalina e carragenina (Damre et al., 2003; Guardia et al., 2003; Abil'daeva et al., 2004; Silvan et al., 1996; Feltenstein et al., 2004). Ademais, apresentam efeito antinociceptivo no modelo de contorções abdominais induzidas por ácido acético (ex. partenolido, costunolido, dehidrocostus lactona) (Jain and Kulkarni, 1999; Okugawa et al., 2000; Ahmed et al., 2001).

A budleína A é uma lactona sesquiterpênica que foi previamente isolada da *Viguiera buddleiaeformis* (de Vivar et al., 1976). Mesmo tendo sido isolada inicialmente na década de 70, existem apenas três estudos *in vitro* demonstrando que a budleína A inibe a mobilidade de espermatozóides (Huacuja et al., 1993), a liberação de elastase por neutrófilos ativados (Arakawa et al., 2008) e a ativação de NF-kB (Siedle et al., 2004). A inibição da ativação do NF-kB pela prevenção da degradação do IkB foi descrita para outras lactonas sesquiterpênicas como isogoiazensolido, centraterina, atripliciolido tiglato (Hehner et al., 1998, Rüngeler et al., 1999; Siedle et al., 2004). A ativação desse fator de transcrição está envolvida na produção de vários mediadores pró-inflamatórios. Após sua ativação, o NF-kB sofre uma translocação do citoplasma para o núcleo celular e induz a expressão de citocinas como o TNFα, IL-1β, IL-6, enzimas como a ciclooxigenase-2 e moléculas de adesão (L-selectinas, ICAM-1) (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996; May and Ghosh, 1998; Barnes et al., 2006), as quais são importantes para a gênese dos sinais cardinais da inflamação.

Neste contexto, Valério et al. (2007) demonstrou *in vivo* que o tratamento por via oral com budleína A inibe a migração de leucócitos, edema e hiperalgesia (resposta exacerbada a um estímulo doloroso) mecânica induzidos pela carragenina e adjuvante completo de Freund. A inibição desses sinais da inflamação pela budleína A está relacionada à inibição da produção de citocinas. Além disso, a budleína A não apresenta efeitos colaterais como lesões gástricas observadas com inibidores da cicloxigenase como a indometacina, e presumivelmente não apresenta efeitos colaterais hormonais como os observados no tratamento crônico com corticosteróides (Valério et al, 2007), pois não agem através da ativação de receptores citoplasmáticos como os corticosteróides, a budleína A deve agir diretamente no NFκB ou IκB (Siedle et al., 2004; Valério et al., 2007). Dessa forma, fica evidente o potencial terapêutico antiinflamatório da budleína A pelo menos em modelos de resposta inflamatória inata como da carragenina e adjuvante completo de Freund (Valério et al., 2007).

Nesse sentido, a budleína A parece ser uma droga com grande potencial terapêutico para doenças crônicas com envolvimento da produção de citocinas como a artrite reumatóide e artrite gotosa.

2. Objetivos

Objetivo Geral

Investigar os efeitos anti-inflamatório e analgésico, e mecanismo de ação da lactona sesquiterpênica budleína A nos modelo de artrite induzida por antígeno e gota em camundongos.

Objetivos Específicos - Artrite induzida por antígeno:

1. Avaliar se o tratamento com budleína A reduz de maneira dose-dependente a hiperalgesia mecânica, edema e migração de leucócitos para a articulação fêmur-tibial induzida pelo desafio com mBSA em camundongos previamente imunizados.
2. Avaliar o efeito do tratamento com budleína A sobre a perda de proteoglicanos induzida pelo desafio com mBSA em camundongos imunizados;
3. Avaliar se o tratamento crônico com budleína A em dose terapêutica induz efeitos tóxicos/adversos através dos níveis de enzimas hepáticas e parâmetros de lesão gástrica;
4. Avaliar se o efeito anti-inflamatório e analgésico da budleína A estaria relacionado a inibição da produção de citocinas pró-inflamatórias induzidas pelo desafio com mBSA em animais imunizados;

Objetivos Específicos - Artrite gotosa:

1. Padronização de dose e tempo para avaliação da inflamação induzida pela administração de cristais de urato monossódico (MSU);

2. Avaliar se o tratamento com budleína A reduz de maneira dose-dependente a inflamação induzida no modelo de gota;
3. Avaliar se o efeito anti-inflamatório e analgésico da budleína A estaria relacionado a inibição da produção de citocinas no modelo de gota;

3.Anexos

ANEXO 1:

The sesquiterpene lactone "budlein A", inhibits antigen-induced arthritis in mice.

The sesquiterpene lactone "budlein A", inhibits antigen-induced arthritis in mice.

[1]Ana C. Zarpelon, [2]Fabricio O. Souto, [2]Larissa G. Pinto, [2]Walter M. Turato, [3]Nilton S. Arakawa, [2]Thiago M. Cunha, [2]Sergio H. Ferreira, [4]João S. Silva, [3]Fernando B. Da Costa, [2]Fernando Q. Cunha, and [1]Waldiceu A. Verri, Jr.

[1]Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid PR 445, Km 380 Cx. Postal 6001, 86051-990, Londrina, Parana, Brazil. Fax: + 55 43 3371-4267, Tel: + 55 43 3371-4387. [2]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil. [3]Department of Pharmaceutical Sciences, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil. [4]Department of Biochemistry and Immunology, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil.

Running title: Budlein A inhibits inflammatory pain.

***Author for correspondence:** Waldiceu A. Verri Jr (waldiceujr@yahoo.com.br or waverri@uel.br). Present address: Departamento de Patologia, Centro de Ciências Biologicas, Universidade Estadual de Londrina. Rod. Celso Garcia Cid Pr 445, KM 380, Cx. Postal 6001, 86051-990, Londrina, Parana, Brazil. Fax: + 55 43 3371-4387, Tel: + 55 43 3371-4979.

Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by articular lesions, recruitment of inflammatory cells and cytokine production. Therefore, corticoids, which diminish cytokine production by inhibition of NFκB activation and/or activity, are therapeutically effective. Nevertheless, although inhibition of NFκB activity is an effective anti-inflammatory mechanism of corticoids, their adverse hormonal effects limit their use. Therefore, a drug that inhibits NFκB activation without the adverse side effects of corticods would be a suitable therapeutic strategy. **Methods:** Antigen-induced arthritis (AIA) was induced in C57BL/6 mice by immunization with 500μg of methylated bovine serum albumine (mBSA) in 0.2ml of an emulsion containing saline and complete Freund's adjuvant subcutaneously (s.c.) on the day 0 and 7, and intraarticular (i.a.) challenge with mBSA (30μg/cavity) on day 21 and 24. Immunized mice were pre-treated with dexamethasone (2mg/Kg) as control and budlein A (1 or 10mg/kg, p.o.) 30 min before intrarticular injection of mBSA and then, every 24 h. Articular hypernociception and edema were determined by electronic pressure meter test and analog caliper, respectively. Leukocyte migration was determined using Neubauer chamber and Rosenfelt stained slices. The proteoglycans dosage was determined by DMMB method, and cytokines by ELISA. To evaluated if the budlein A induces liver and stomach damage we performed the ALT, AST and myeloperoxidase activities. **Results:** The oral treatment with budlein A for 6 days dose (1 and 10 mg/kg)-dependently inhibited mBSA-induced mechanical hypernociception, oedema, total leucocytes, neutrophils recruitment and proteoglycans loss. Cytokine

production in the spinal cord was also reduced in the 24th day. There was no detectable side effect such as gastric lesions or liver damage as addressed by ALT and AST levels, due to budlein A treatment. **Conclusions:** These results suggested that budlein A treatment might be a suitable approach in rheumatoid arthritis therapy by inhibiting inflammation-induced cytokine production with reduced side effects.

Keywords: budlein A, rheumatoid arthritis, pain, inflammation.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with 1% prevalence in the modern world. It comprises a syndrome of pain, stiffness, and symmetrical synovitis (inflammation of the synovial membrane). RA is characterized by an increase in infiltration of immune cells (including neutrophils, T cells, B cells and macrophage) into the synovial membrane, cavity and periarticular tissues, and the effector function of each of these cells is orchestrated mainly by a network of cytokines such as TNFα and IL-1β (Pinto et al., 2010; Harris et al., 1990; Brackertz et al., 1977; Gabay and McInnes, 2009; Robert et al., 2009).

One of the most prevalent symptoms of RA is the increase in the sensitivity to joint pain due to nociceptor sensitization, which usually leads to clinical conditions known as hyperalgesia (an increased response to a stimulus that is normally painful) that cause movement limitations (Verri et al., 2006). In RA both thin myelinated (Aδ fibers) and unmyelinated (C fibers) neurons become sensitized and start to respond to light pressure and movements in the working range of the joint. Finally, mechanoinsensitive fibers (silent nociceptors) become responsive to mechanical stimulation of the joint and contribute to the afferent inflow into the spinal cord during inflammation. The consequence of these processes is that under inflammatory conditions the nociceptive system is activated by the normally innocuous and nonpainful mechanical stimuli (Schaible et al., 2002).

The experience of pain is generally associated with enhance release of pro-inflammatory cytokines, which in turn sensitize the nociceptors, promoting further amplification of pain transmission (De Jongh et al., 2003). In this sense,

cytokine targeting therapies such as anti-TNF and anti-IL-1 inhibit arthritis inflammation. Immunosuppressive drugs such as glucocorticoids, which inhibit cytokine production by inhibition of NFκB activation and/or activity is also effective, however their adverse hormonal effects limit their use (Baeuerle et al., 1994; Barnes et al., 2006).

In addition to the autoimmune characteristics of RA, there is also evidence that toll-like receptors (TLR) are expressed and activated within the joint in RA. For instance, TLR3 is activated by necrotic synoviocytes (Brentano et al., 2005), and TLR2 and TLR4 are activated by unknown endogenous ligands, and experimentally activated by lipotheicoic acid and lypopolysaccharide (LPS), respectively. The TLR activation also results in NFκB activation and cytokine production (Liu-Bryan et al., 2005).

Therefore, a drug that inhibits NFκB activation without the adverse side effects of glucocorticods would be a suitable therapeutic strategy. In this sense, budlein A, a sesquiterpene lactone obtained from *Viguiera robusta*, seems a promising drug. Budlein A (Fig. 1) has been previously isolated from *Viguiera buddleiaeformis* (De Vivar et al., 1976) and *Viguiera arenaria* (Siedle et al., 2004), however, despite its first isolation in the seventies, there are few reports on its activities. Budlein A inhibits in vitro sperm motility (Huacuja et al., 1993), TNFα-induced NFκB activation in Jurkat cells as shown by the eletrophoretic mobility shift assay (Siedle et al., 2004). In vivo, we have shown that it inhibits carrageenin-induced paw inflammation by inhibiting cytokine production (Valerio et al., 2007). Our data on inhibition of leukocyte recruitment and cytokine production (Valerio et al., 2007) was reinforced by others, which also

demonstrated reduction of adhesion molecules by budlein A (Nicolete et al., 2009).

In the present study, it was addressed whether budlein A treatment would be a promising therapeutic strategy in a model of rheumatoid arthritis in mice. Budlein A treatment inhibited arthritis-induced knee pain, edema, leukocyte recruitment, proteoglycan loss and cytokines production.

Materials and Methods

Animals

The experiments were performed on male C57BL/6 weighing between 20 and 25g. The mice were housed in temperature-controlled rooms (22-25C), with access to water and food *ad libitum*. All experiments were conducted in accordance with animals' care and handling procedures of the International Association for Study of Pain (IASP) and with the approval of the Ethics Committee (protocol no 05761) of the Universidade Estadual de Londrina.

Plant Material

The *Viguiera robusta* were collected in Batatais, state of São Paulo in April 2001 by F.B.C. In Biology Institute, Universidade de Uberlândia, Uberlândia, MG, Brazil were identified the material. Avoucher specimen (FBC#105) is deposited at the herbarium of the Department of Biology, University of São Paulo, Ribeirão Preto, São Paulo, Brazil under the code SPFR 07155. (Da Costa et al., 2001) Its chemical structure was determined by means of spectrometric analysis, i.e. IR and nuclear magnetic resonance (NMR) spectrometry (^{1}H and ^{13}C), as well as comparison with authentic sample and data reported in the literature (Da Costa et al., 2001). The purity of budlein A was determined by chromatographic and spectrometric methods. TLC was carried out using several eluent systems and two spray reagents (1% vanillin–sulphuric acid or concentrated sulphuric acid).Ahigh performance liquid chromatography (HPLC) run was made using methanol–water 55:45 or acetonitrile–water 65:35 as mobile phase, a reversed phase (ODS) analytical

column, flowrate 1.0 or 1.3 mL/min, and UV detection at λmax 225 and 265 nm, as described elsewhere (Da Costa et al., 2001). Only one compound was detected in the chromatographic analyses. The 13C NMR spectrum of budlein A showed 20 carbon atoms corresponding to its structure. By means of chromatographic and spectrometric methods,we estimated that the purity of budlein A is between 95–98%, therefore suitable for these biological assays.

Drugs and stimuli

Dexamethasone, methylated bovine serum albumin (mBSA) and complete Freund's adjuvant (CFA) were obtained from Sigma-Aldrich, St. Louis, MO, USA. The 1,9-Dimethylmethylene blue (DMMB) was purchased from Polysciences, Inc and the chondroitin 4-sulphate sodium salt utilized as standard was purchased from Biochemika.

Induction of Experimental Arthritis – Antigen-induced arthritis

Mice were immunized as described previously (Verri et al., 2008). C57BL/6 mice were immunized with 500 μg of methylated bovine serum albumin (mBSA) in 0.2 ml of an emulsion containing saline and complete Freund's adjuvant (CFA – 1 mg/ml of *Mycobacterium tuberculosis*, in mineral oil) by subcutaneous (s.c.) route on the day 0 and 7. AIA was induced by intraarticular (i.a.) injection of mBSA (30μg/cavity) on day 21 and 24. Sham-immunized mice were given similar injections but without the antigen (mBSA).

Evaluation of articular hypernociception

The articular hypernociception of the femur-tibial joint evaluated by electronic

von Frey apparatus. For this, mice were placed in acrylic cages a wire grid floor, and the stimulations were performed only when the animals were quiet (and with the four paws on the grid floor). This method consists of an electronic pressure-meter, with a force transducer fitted with polypropylene tip (Insight instruments, Ribeirao Preto, SP, Brazil). To evaluate the articular pain, we used a large tip (4.15mm^2), to exclude the subcutaneous effect (Guerreiro et al., 2008). An increase perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the femur-tibial joint followed of the hind paw withdrawal. A digital analgesymeter recorded the intensity of the force applied when the paw was withdrawn, expressed in grams (g). The test was performed in the times indicated on figures.

Paw oedema test

The volume of the mice joint was measure with a caliper (Mitutoyo) before (zero time) the intraarticular stimulus with mBSA, and after the administration, in the times indicated. The amount of paw swelling was determined for each mouse and the difference between the times indicated on figures and the zero time. The oedema value was represented by oedema/mm.

Proteoglycans assay

Patellae were fixed in 10% formalin overnight, decalcified in formic acid (5%) for 4 hours, and subsequently digested overnight at 60 °C in 60 µl of 10 mg/ml papain (type IV; Sigma) in 0.1 M sodium acetate, pH 6.5, 10 mM L-cysteine, 50 mM disodium EDTA per patella. After the digestion, the samples were centrifuged at 1500 rpm for 10 min and were ready for the GAG measurement. The assay was calibrated by use of reagent blanks, and standards containing

up to 5 µg of chondroitin sulfate in the same solvent as the samples. The measurement was realized 30 s after the color reagent addition, at 525 nm in a Microplate Spectrophotometer System – SpectraMax (Molecular Devices) (Verri et al., 2010).

In vivo neutrophil migration

Immunized or sham-immunized mice had mBSA or vehicle (saline) injected directly into the articular cavity (Lemos et al., 2009; Verri et al, 2010). At the time-points indicated on figures, the articular cavities were washed 3 times with 3.3µL of saline with 1mM EDTA. The total number of neutrophils was determined in a Neubauer chamber diluted in Turk's solution 1:2 (used to lyse the erythrocytes). Differencial cell counts were determined by Roselfeld stained slices using a light microscope and results were expressed as the number of neutrophils per cavity.

Biochemical Analysis

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were determined using commercial kits (Labtest – Lagoa Santa, Minas Gerais, Brazil) according to the manufecture's instructions.

Cytokines measurement

After the i.a. injection of the inflammatory stimuli (mBSA), animals were terminally anesthetized and the spinal cord (L3, L4 and L5) were removed and homogenized in 200µL of buffer. TNFα and IL-1β concentration were

determined by ELISA (Ebioscience, 88-7324-76 and 88-7013-76 respectively). The results were expressed as pg/mg of tissue.

Myeloperoxidade Activity

Myeloperoxidase (MPO) activity was used as an index of neutrophil accumulation in the mice stomachs tissue, based on a kinetic-colorimetric assay as described previously (Bradley et al., 1982; Casagrande et al., 2006). Approximately 0.5 cm^2 of stomach tissue was collected after the second challenge. Samples were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide and kept at -80°C. Just before the assay, the tissue was homogenized using a Polytron (PT3100) and centrifuged at 13.000 *g* for 2 min. To prepare the solution for the analysis, 10 µl supernatant was mixed with 200 µl 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The solution was analyzed by spectrophotometry for MPO activity determination at 450 nm (Spectra max) with three readings in 1 min. The MPO activity was compared with a standard curve of neutrophils. The results were presented as number of neutrophils x 10^6/mg tissue.

Experimental protocol

Mice were treated with budlein A (1 and 10mg/kg, 30 min, diluted in 20% of Tween 80 plus saline, to complete 150 µL, p.o. pathway) before stimulus with mBSA (30 µg/i.a.) on day 21, and further treatments were performed at 24 h intervals up to 26 days after mBSA challenge. Mechanical hypernociception and oedema were evaluated 1, 3, 5, 12, 24 and 48h after the first and second

challenge. The neutrophil migration and proteoglycans were evaluated after the second challenge. Possible budlein A-induced liver or stomach damage were evaluated using blood samples to determine the levels of ALT and AST, and stomach samples to determine the myeloperoxidase activity. The spinal cord were removed and homogenized in 200 µL of saline, TNFα and IL-1β concentration were determined by ELISA assay.

Statistical analyses

The results are representative of two independent experiments and are presented as the mean ± standard error mean (s.e.m.). One-way ANOVA followed by Bonferroni's *t*-test was performed to evaluate the differences between responses, on Graph Prism program. Statistical differences were considered to be significant at $P < 0.05$.

Results

Budlein A inhibits in a dose-dependent manner the inflammation in antigen-induced arthritis.

In the first series of experiments, it was addressed whether budlein A inhibits mechanical hypernociception and oedema induced by mBSA (i.art.) challenge. Mice were treated with dexamethasone (2mg/Kg, s.c.), budlein A (1 and 10mg/Kg) or vehicle (20% of Tween 80 plus saline to complete 150 µL, p.o.) 30 min before mBSA stimuli (30 µg/i.art., day 21) and at 24 h intervals from day 21 to 26. The intensity of mechanical hypernociception (Fig. 1A and 1B) and edema (Fig. 1C and 1D) were evaluated after first challenge (Fig. 1A and 1C) and second challenge (Fig. 1B and 1D) at indicated time points. At the 26th day, knee joints cells were harvested for total leukocytes (Fig. 1E) and neutrophil counts (Fig. 1 F). Budlein A treatment significantly and dose-dependently reduced antigen challenge-induced intensity of hypernociception, oedema and total leukocytes and neutrophil recruitment to the joints. So, we concluded that budlein A inhibited the major signs of inflammation, such as pain (demonstrated by intensity of hypernociception), oedema and leucocytes counts. The control drug dexamethasone also inhibited all parameters addressed.

The Budlein A inhibits the proteoglycans lose

The proteoglycans dosage was performed in knee joint samples because its content reflects joint damage. The budlein A treatment (1 and 10mg/Kg, p.o.) also inhibited antigen challenge-induced proteoglycan loss in knee joint samples (Fig. 2), but only 10mg/Kg dose inhibited the proteoglycans loss. Theregore, we are demonstrating that budlein A reduces tissue lesions.

Although, corticosteroids clinically prevent cartilage destruction, the dexamethasone dose regimen used in the present study was not able to reduce proteoglycan loss (Fig. 2).

The Budlein A did not cause liver and stomach damage

An important issue to be addressed is whether novel drugs present adverse side effects. We focused in two main relevant side effects; gastric lesions and liver function. For these purposes, samples of the stomach were collected for determination of myeloperoxidase (MPO) activity. It is well established that gastric lesions are accompanied by increase of MPO activity reflecting neutrophil influx (Valerio et al., 2007). There was no increase of MPO activity (Fig. 3A), indicating budlein A does not induce gastric lesions. Liver function is routinely determined by the levels of ALT and AST from blood samples. Again, there were no alterations on the enzyme blood levels by budlein A treatment (Fig. 3B and 3C).

Budlein A inhibits cytokine production (TNFα and IL-1β)

The spinal cord cytokine production is an important contributing feature to hypernociception, therefore, the levels of TNFα and IL-1β in the spinal cord tissue were evaluated. Mice were treated with budlein A (10mg/Kg, p.o., 30 min) or vehicle (20% of Tween 80 plus saline to complete 150 µL) 30 min before mBSA (30µg/i.a.) stimulus at day 21 and at 24 h intervals up to 24th day when spinal cord samples between L4-L6 were collected for cytokine level determination by ELISA. Budlein significantly reduced TNFα and IL-1β production in the spinal cord in mBSA-induced arthritis (Fig 4 A and 4B).

Discussion

The budlein A is a sesquiterpene lactone that inhibits carrageenin-induced inflammation (Valerio et al., 2007). In the present study, it was demonstrated that budlein A inhibits the development of inflammation in a mice model of rheumatoid arthritis (RA), including relevant clinical sign such as edema, pain and cartilage destruction, by a mechanism related to inhibition of cytokine production (such as TNFα and IL-1β).

RA is a chronic auto-immune disease with joint pain, oedema, influx of inflammatory cells, bone and cartilage destruction that ultimately leads to loss of function that debilitates the RA patients. Disease modifying antirheumatic drugs (DMARDs) such as metotrexate and hydroxychloroquine, immunesuppressors such as corticoids, and biological therapies including anti-TNF antibodies and soluble receptors are the frontline strategies to reduce disease progression and avoid debilitation of RA patients.

The current therapeutic strategies for RA include combined treatment with a non biological DMARDs or corticosteroid with an immune biological. In fact, the combination of metotrexate plus anti-TNF therapies is a current standard approach for RA. However, one significant adverse side effect observed is the increase of susceptibility to bacterial and viral infections (Brennan & McInnes, 2008; Verri et al., 2010). In the case of corticosteroids, they are very powerful anti-inflammatory drugs that act in a corticoid receptor-dependent manner to inhibit the transcription of proinflammatory molecules by NFκB, and to induce IκB expression that inhibits NFκB activity, and production of anti-inflammatory molecules expression such as IL-10. The use of corticosteroids is limited by their side effects related to the corticoid receptor-

dependent hormonal activity (Barnes et al, 2006). Nevertheless, inhibition of NFκB activation without the hormonal side effects seems a promising strategy to reduce RA inflammation.

In agreement, the present data demonstrated that budlein A reduced the inflammation in a model of sub-chronic RA, including reduced joint pain, edema, leukocyte recruitment (Fig. 1), and even reduced loss of proteoglycans in patellae samples (Fig. 2).

In general, cytokine production is evaluated in the periphery, but its spinal production is begging to be clearly related to pain development in RA and other conditions (Gao & Ji, 2010). Further supporting that budlein A has therapeutic potential, it inhibited the production of spinal TNFα and IL-1β induced in RA. We previously demonstrated that budlein A diminishes inflammatory overt pain-like behavior in models such as acetic acid-induced writhing response and formalin-induced flinches (Valerio et al., 2007). Furthermore, budlein A inhibits carrageenin-induced paw edema, neutrophil recruitment, and mechanical hypernociception by a mechanism related to reduction of TNFα, IL-1β and CXCL1 production in the paw tissue (Valerio et al., 2007). Additionally, it was observed that budlein A also inhibits proteinase secretion by neutrophils (Arakawa et al., 2008), which could account for the reduction of proteoglycan loss by budlein A treatment in RA (present data).

Corroborating the present data, it was demonstrated that stimulation of Jurkat cells with the inflammatory mediator TNFα induces NFκB activation, which was inhibited by budlein A in electrophoretic mobility shift assay, confirming that budlein is really able to inhibit NFκB activation (Siedle et al., 2004). Moreover, extracts from plants rich in sesquiterpene lactones have

gained considerable interest for treating human diseases, including cancer. The sesquiterpene lactones-derived drugs from thapsigargin, artemisinin, and parthenolide are now in cancer clinical trials (Ghantous et al, 2010).

Conclusions

It was reported that the treatment with budlein A, a sesquiterpene lactone from *Viguiera robusta*, significantly reduced the inflammation in a model of RA. There was reduction of joint pain, edema, leukocyte recruitment and prevention of proteoglycan loss. The mechanism underlying this anti-inflammatory effect of budlein A was related to inhibition of cytokine production in important sites such as spinal cord.

List of Abbreviations: Rheumatoid arthritis (RA), methylated bovine serum albumine (mBSA), complete Freund's adjuvant (CFA), sesquiterpenes lactones (STLs), *Viguiera robusta (V. robusta)*, grams (g), intraarticular injection (i.a.), Orally via (p.o.), Subcutaneous via (s.c.), myeloperoxidase (MPO), Tumor necrosis factor alpha (TNFα), Lipopolysaccharide of gram negative bacteria (LPS), Interleukin-1 beta (IL-1β), Keratinocyte-derived chemokine (KC/CXCL1).

Competing Interests

The authors declare that they have no competing interests.

Acknowledgment and Funding

This work was supported by grants from Conselho Nacional de Pesquisa (CNPq, Brazil), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Fundação Araucária. Ana Carla Zarpelon was a fellow of CNPq, Brazil.

References

Abil'daeva AZ, Pak RN, Kulyiasov AT, Adekenov SM (2004) Anti-inflammatory effect of arglabin and 11,13-dihydro-13-dimethylaminoarglabin hydrochloride. Eksp Klin Farmakol 67:37-39.

Ahmed M, Rahman MT, Alimuzzaman M, Shilpi JA (2001) Analgesic sesquiterpene dilactone from Mikania cordata. Fitoterapia 72:919-921.

Arakawa NS, Schorr K, Ambrósio SR, Merfort I, Da Costa FB (2008) Further sesquiterpene lactones from Viguiera robusta and the potential anti-inflammatory activity of a heliangolide: inhibition of human neutrophil elastase release. Z Naturforsch [C] 63(7-8):533-538.

Baeuerle PA, Baltimore D (1996) NF-kappa B: ten years after. Cell 87:13-20.

Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141-179.

Barnes PJ (2006) How corticosteroids control inflammation: Quintiles Prize Lecture 2005 Br J Pharmacol 148:245-254.

Brackertz D, Mitchell GF, Vadas MA, Mackay IR, Miller JF (1977) Studies on antigen-induced arthritis in mice. II. Immunologic correlates of arthritis susceptibility in mice. J Immunol 118:1639-1644.

Casagrande R, Georgetti SR, Verri WA Jr, Dorta DJ, dos Santos AC, Fonseca MJ (2006). Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. J Photochem Photobiol 3; 84(1): 21-7.

Conte FD, Barja-Fidalgo C, Verri WA, Jr, Cunha FQ, Rae GA, Penido C and Henriques MD (2008) Endothelins modulate inflammatory reaction in zymosan-induced arthritis: participation of LTB4, TNF-α, and CXCL-1. J Leukoc Biol 84:652-660.

Cunha TM, Verri WA Jr, Valério DA, Guerrero AT, Nogueira LG, Vieira SM, Souza DG, Teixeira MM, Poole S, Ferreira SH, Cunha FQ (2008) Role of cytokines in mediating mechanical hypernociception in a model of delayed-type hypersensitivity in mice. Eur J Pain 12(8):1059-1068.

Cunha TM, Verri WA, Jr, Vivancos GG, Moreira IF, Reis S, Parada CA, Cunha FQ, Ferreira SH (2004) An electronic pressure-meter nociception paw test for mice. Braz J Med Biol Res 37:401-407.

Damre AA, Damre AS, Saraf MN (2003) Evaluation of sesquiterpene lactone fraction of Saussurea lappa on transudative, exudative and proliferative phases of inflammation. Phytother Res 17:722-725.

Da Costa FB, Schorr K, Arakawa NS, Schilling EE, Spring O. (2001).

Infraspecific variation in the chemistry of glandular trichomes of two Brazilian Viguiera populations. J. Braz. Chem. Soc. 12, 403-407.

De Jongh RF, Vissers KC, Meert TF, Booij LH, De Deyne CS, Heylen RJ: The role of interleukin-6 in nociception and pain. *Anesth Analg* 2003, 96: 1096-103, table.

De Vivar AR, Diaz CGE, Bratoeff EA, Jimenez L (1976) The germacranolides of Viguiera buddleiaeformis structures of budlein-A and –B. Phytochemistry 15:525-529.

Feltenstein MW, Schuhly W, Warnick JE, Fischer NH, Sufka KJ (2004) Anti-inflammatory and anti-hyperalgesic effects of sesquiterpene lactones from Magnolia and Bear's foot. Pharmacol Biochem Behav 79:299-302.

Gabay C and McInnes IB (2009). The biological and clinical importance of new generation cytokines in rheumatic diseases. Arthritis Research & Therapy, 11:230

Ghantous A, Gali-Muhtasib H, Vuorela H, Saliba NA, Darwiche N (2010). What made sesquiterpene lactones reach cancer clinical trials? Drug Discovery Today, 15: 16/24.

Grespan R, Fukada SY, Lemos HP, Vieira SM, Napimoga MH, Teixeira MM, Fraser AR, Liew FY, McInnes IB, Cunha FQ (2008) CXCR2-specific

chemokines mediate leukotriene B(4)-dependent recruitment of neutrophils to inflamed joints in mice with antigen-induced arthritis. Arthritis and rheumatism 58(7):2030-2040.

Guardia T, Juarez AO, Guerreiro E, Guzman JA, Pelzer L (2003) Anti-inflammatory activity and effect on gastric acid secretion of dehydroleucodine isolated from Artemisia douglasiana. J Ethnopharmacol 88:195-198.

Guerrero AT, Verri WA, Jr., Cunha TM, Silva TA, Rocha FA, Ferreira SH, Cunha FQ and Parada CA (2006) Hypernociception elicited by tibio-tarsal joint flexion in mice: a novel experimental arthritis model for pharmacological screening. Pharmacol Biochem Behav 84:244-251.

Guerrero AT, Verri WA, Jr., Cunha TM, Silva TA, Schivo IR, Dal-Secco D, Canetti C, Rocha FA, Parada CA, Cunha FQ and Ferreira SH (2008) Involvement of LTB4 in zymosan-induced joint nociception in mice: participation of neutrophils and PGE2. J Leukoc Biol 83:122-130.

Harris Jr ED. Rheumatoid arthritis. Pathophysiology and implications for 724 therapy. N Engl J Med 1990;322:1277–89.

Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, Schulze-Osthoff K, Droge W, Schmitz ML (1998) Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. J Biol Chem 273:1288-1297.

Huacuja RL, Carranco A, Guzman SA, Guerrero C (1993) Inactivation of SH groups with sesquiterpene lactones: effects on nuclear decondensation pattern / motility induced by heparin in human spermatozoa. Adv Contracept Deliv Syst 9:97-106.

Jain NK, Kulkarni SK (1999) Antinociceptive and anti-inflammatory effects of Tanacetum parthenium L. extract in mice and rats. J Ethnopharmacol 68:251–259.

Lemos HP, Grespan R, Vieira SM, Cunha TM, Verri WA, Jr, Fernandes KS, Souto FO, McInnes IB, Ferreira SH, Liew FY, Cunha FQ (2009) Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN production. Proc Natl Acad Sci USA.

Liu-Bryan R, Scott P, Sydlaske A, Rose DM, Terkeltaub R. (2005) Innate Immunity Conferred by Toll-like Receptors 2 and 4 and Myeloid Differentiation Factor 88 Expression Is Pivotal to Monosodium Urate Monohydrate Crystal–Induced Inflammation. Arthritis & Rheumatism, 52: (9) pp 2936–2946.

May MJ, Ghosh S (1998) Signal transduction through NF-kappa B. Immunol Today 19:80-88.

Meshnick SR (2002). Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 32:1655–1660.

Nicolete R, Arakawa NS, Rius C, Nomizo A, Jose PJ, Da Costa FB, Sanz MJ, Faccioli LH. (2009). Budlein A from Viguiera robusta inhibits leukocyte-endothelial cell interactions, adhesion molecule expression and inflammatory mediators release. Phytomedicine. 16(10):904-15. Epub 2009 Jun 12.

Okugawa H, Ueda R, Matsumoto K, Kawanishi K, Kato K (2000) Effects of sesquiterpenoids from "Oriental incenses" on acetic acid-induced writhing and D2 and 5-HT2A receptors in rat brain. Phytomedicine 7:417-422.

Pickman AK (1984) Antifungal activity of sesquiterpene lactones. Biochem Syst Ecol 12:13–18.

Pinto LG, Cunha TM, Vieira SM, Lemos HP, Verri WA Jr., Cunha FQ, Ferreira SH (2010). IL-17 mediates articular hypernociception in antigen-induced arthritis in mice. Pain, 148 (2):247-56.

Robert R Edwards, Ajay D Wasan, Clifton O Bingham III, Joan Bathon, Jennifer A Haythornthwaite, Michael T Smith, Gayle G Page: Enhanced reactivity to pain in patients with rheumatoid arthritis. *Arthritis Research & Therapy* 2009, **11**:R61

Rocha FA, Leite AK, Pompeu MM, Cunha TM, Verri WA, Jr, Soares FM, Castro RR, Cunha FQ (2008) Protective effect of an extract from Ascaris suum in experimental arthritis models. Infect Immun 76(6):2736-2745.

Rüngeler P, Castro V, Mora G, Gören N, Vichnewski W, Pahl HL, Merfort I, Schmidt TJ (1999) Inhibition of Transcription Factor NF-kB by Sesquiterpene Lactones: a Proposed Molecular Mechanism of Action. Bioorg Med Chem 7:2343-2352.

Santodomingo-Garzón T, Cunha TM, Verri WA Jr, Valério DA, Parada CA, Poole S, Ferreira SH, Cunha FQ (2006) Atorvastatin inhibits inflammatory hypernociception. Br J Pharmacol 149(1):14-22.

Siedle B, Garcia-Pineres AJ, Murillo R, Schulte-Monting J, Castro V, Rungeler P, Klaas CA, Da Costa FB, Kisiel W, Merfort I (2004) Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappaB. J Med Chem 47:6042-6054.

Silvan AM, Abad MJ, Bermejo P, Villar A (1996) Inhibition by hydroxyachillin, sesquiterpene lactone from Tanacetum microphyllum, of PMA-induced mouse ear oedema. Inflamm Res 45:289-292.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23:479-490.

Valerio DA, Cunha TM, Arakawa NS, Lemos HP, Da Costa FB, Parada CA,

Ferreira SH, Cunha FQ and Verri WA, Jr. (2007) Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: inhibition of cytokine production-dependent mechanism. Eur J Pharmacol 562:155-163.

Verri WA Jr, Cunha TM, Parada CA, Poole S, Cunha FQ, Ferreira SH (2006). Hypernociceptive role of citokines and chemokines: targets for analgesic drug development: Pharmacol. Ther. 112 (1): 116-38.

Verri WA, Jr, Souto FO, Vieira SM, Fukada SY, Xu D, Alves-Filho JC, Cunha TM, Guerrero AT, Almeida SC, Oliveira FR, Louzada-Junior P, Teixeira MM, Silva JS, McInnes IB, Ferreira SH, Liew FY, Cunha FQ (2010) IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. J Clin Invest.

Verri, W.A., Jr., A.T. Guerrero, S.Y. Fukada, D.A. Valerio, T.M. Cunha, D. Xu, S.H. Ferreira, F.Y. Liew, and F.Q. Cunha. (2008). IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. Proc. Natl. Acad. Sci. U S A 105:2723-2728.

Xu D, Jiang HR, Kewin P, Li Y, Mu R, Fraser AR, Pitman N, Kurowska-Stolarska M, McKenzie AN, McInnes IB, Liew FY (2008) IL-33 exacerbates antigen-induced arthritis by activating mast cells. Proc Natl Acad Sci U S A 105:10913-10918.

Legends to figures

Figure 1: Budlein A inhibits antigen-induced articular inflammation in a dose-dependent manner. Mice were treated with dexamethasone (2 mg/kg, s.c.), budlein A (1 and 10mg/Kg, 30 min) or vehicle (saline or 20% of Tween 80 plus saline to complete 150 µL, p.o.) 30 min before mBSA (30 µg/joint) stimulus on day 21 and up to day 26 in 24 h intervals. The intensity of mechanical hypernociception (Panel A and B) and edema (Panel C and D) were evaluated 1,3,5,7, 24 and 48h after first (Panel A) and in the same time points after the second (Panel B) challenge with mBSA using electronic von Frey and caliper, respectively. In the 26^{th} day, mice were sacrificed and the knee joint cavities were washed 3 times with 3.3µL of saline with 1mM EDTA. The total number of neutrophils was determined in a Neubauer chamber diluted in Turk's solution 1:2 (Panel E). Differencial cells counts were determined by Roselfelt stained slices using a light microscope and results were expressed as the number of leukocytes and neutrophils per cavity (Panel E and F). *P<0.05 compared with the negative control – saline + vehicle (indicated as zero), #P<0.05 compared with positive + vehicle control group. **P<0.05 compared with Budlein dose of 10 mg/Kg (p.o.). One-way ANOVA followed by Bonferroni's test.

Figure 2: Budlein A inhibits antigen-induced arthritis knee proteoglycans loss. Mice were treated with dexamethasone (2 mg/kg s.c.), budlein A (1 and 10 mg/Kg, 30 min) or vehicle (20% of tween 80 saline to complete 150 µL, p.o. via) 30 min before mBSA (30 µg/i.a. on day 21) stimulus, and in 24 h intervals up to day 26. *P<0.05 compared with the negative control – saline + vehicle,

#*P*<0.05 compared with positive (mBSA group). One-way ANOVA followed by Bonferroni's test.

Figure 3: The budlein A did not cause liver and stomach damage. Mice were treated with dexamethasone (2 mg/kg, s.c.), budlein A (1 and 10 mg/Kg, 30 min) or vehicle (20% of tween 80 saline to complete 150 µL, p.o. via) 30 min before mBSA (30 µg/i.a., on day 21) stimulus and in the following days in 24 h intervals up to day 26. At the end of treatment, samples of stomach tissue were analysed for myeloperoxidase activity to determine local inflammation (Panel A). Blood samples were collected for biochemical tests (ALT, AST) to address liver function. None of the treatments induced significant alteration.

Figure 4: Budlein A inhibits TNFα and IL-1β production. Mice were treated with budlein A (1 and 10 mg/Kg, 30 min) or vehicle (20% of tween 80 saline to complete 150 µL, p.o. via) 30 min before mBSA (30 µg/i.a, on day 21), and in the following days in 24 h intevals. Spinal cord were collected for TNFα (Panel A) and IL-1β (Panel B) determination by ELISA on day 23. **P*<0.05 compared with the negative control – saline + vehicle (indicated as zero), #*P*<0.05 compared with positive + vehicle control group. One-way ANOVA followed by Bonferroni's t test.

Figure 1

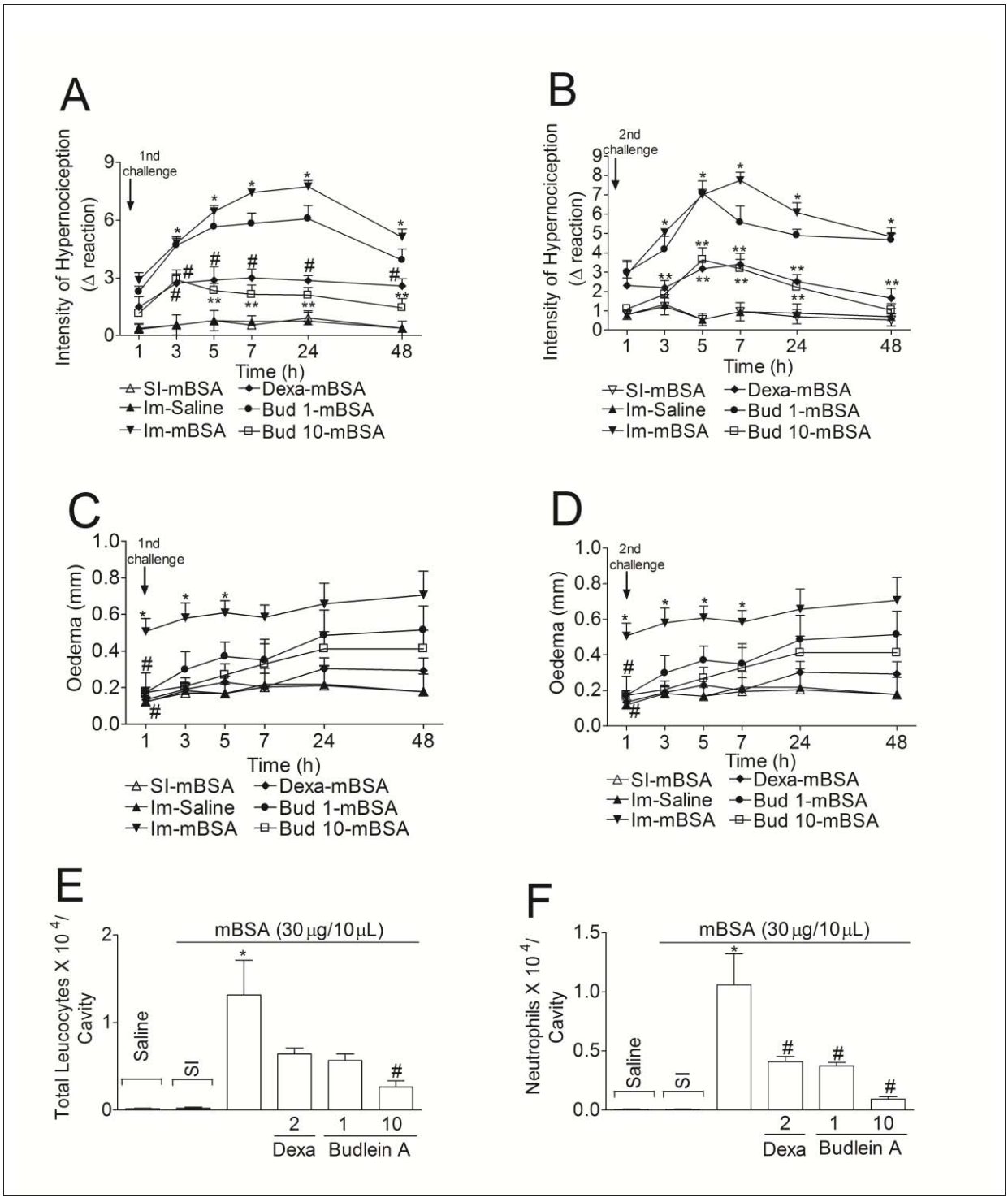

A
1nd challenge
Intensity of Hypernociception (Δ reaction)
Time (h)
SI-mBSA
Im-Saline
Im-mBSA
Dexa-mBSA
Bud 1-mBSA
Bud 10-mBSA
B
2nd challenge
Intensity of Hypernociception (Δ reaction)
Time (h)
SI-mBSA
Im-Saline
Im-mBSA
Dexa-mBSA
Bud 1-mBSA
Bud 10-mBSA
C
1nd challenge
Oedema (mm)
Time (h)
SI-mBSA
Im-Saline
Im-mBSA
Dexa-mBSA
Bud 1-mBSA
Bud 10-mBSA
D
2nd challenge
Oedema (mm)
Time (h)
SI-mBSA
Im-Saline
Im-mBSA
Dexa-mBSA
Bud 1-mBSA
Bud 10-mBSA
E
mBSA (30 μg/10 μL)
Total Leucocytes X 10 4/ Cavity
Saline
SI
2
1
10
Dexa
Budlein A
F
mBSA (30 μg/10 μL)
Neutrophils X 10 4/ Cavity
Saline
SI
2
1
10
Dexa
Budlein A

Figure 2

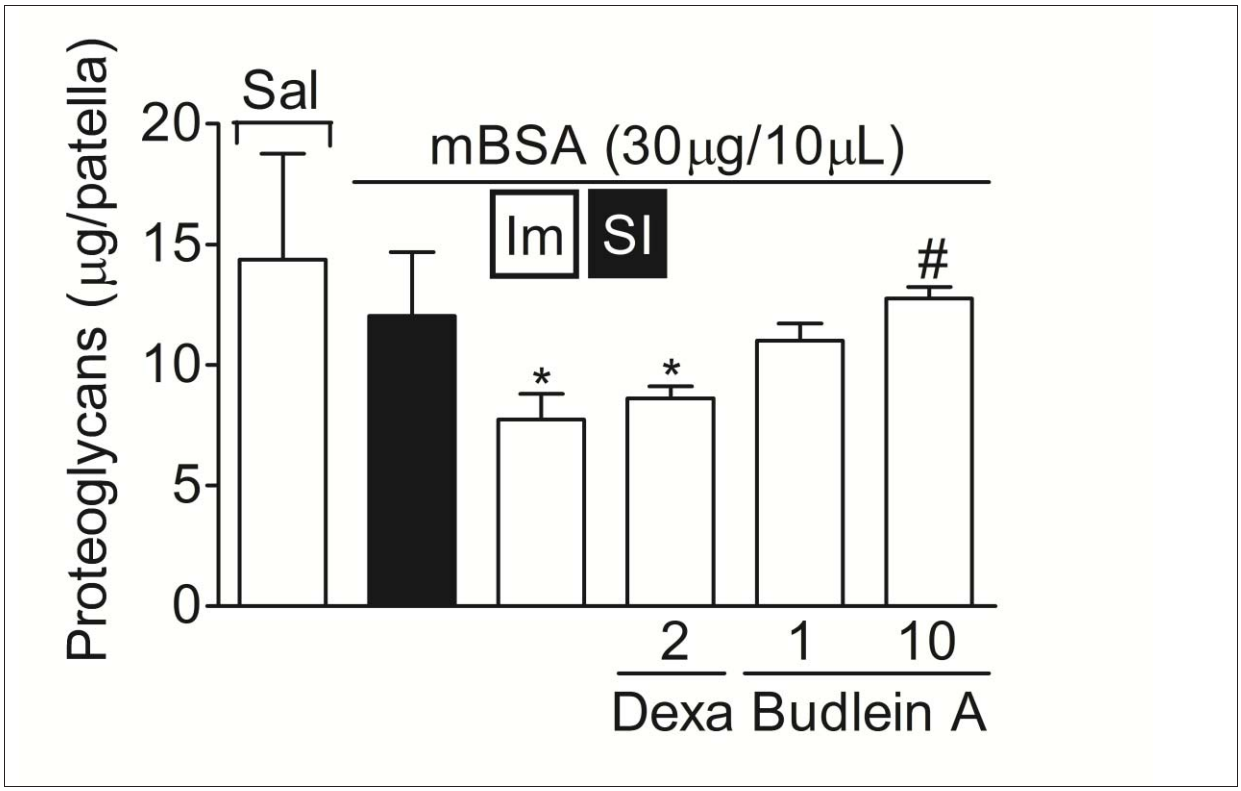

Sal
mBSA (30μg/10μL)
Im
Sl
Proteoglycans (μg/patella)
20
15
10
5
0
*
*
#
2
1
10
Dexa
Budlein A

Figure 3

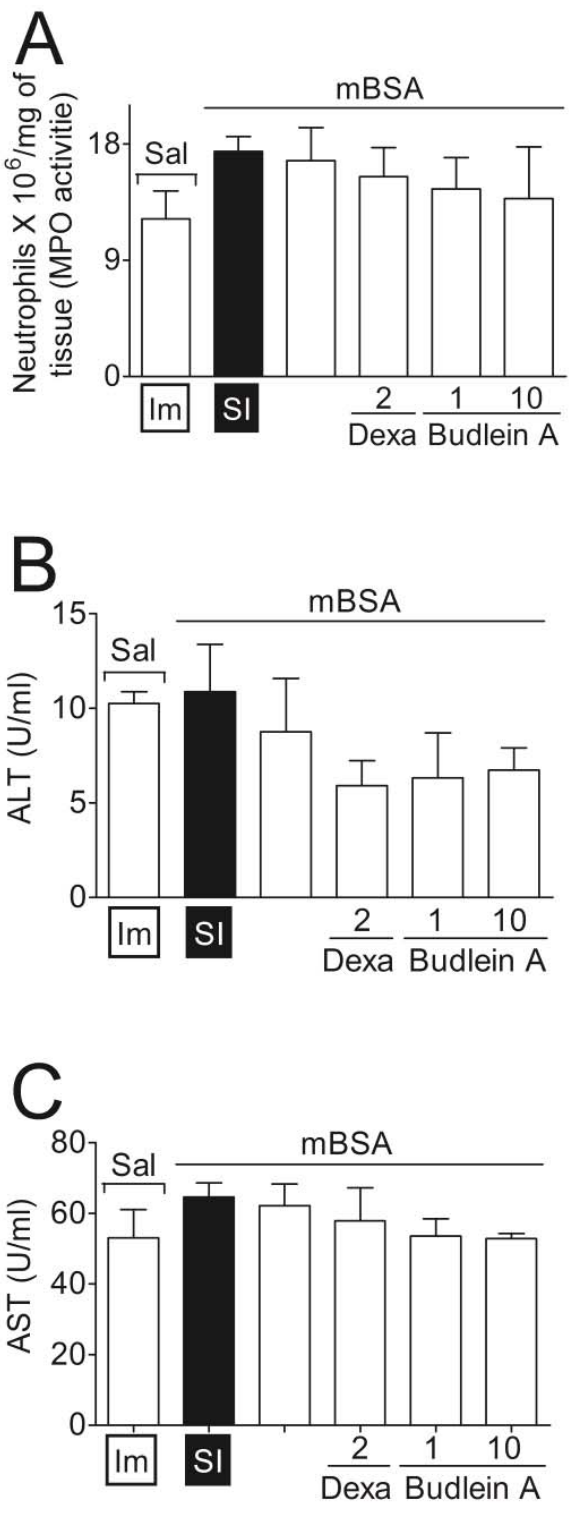
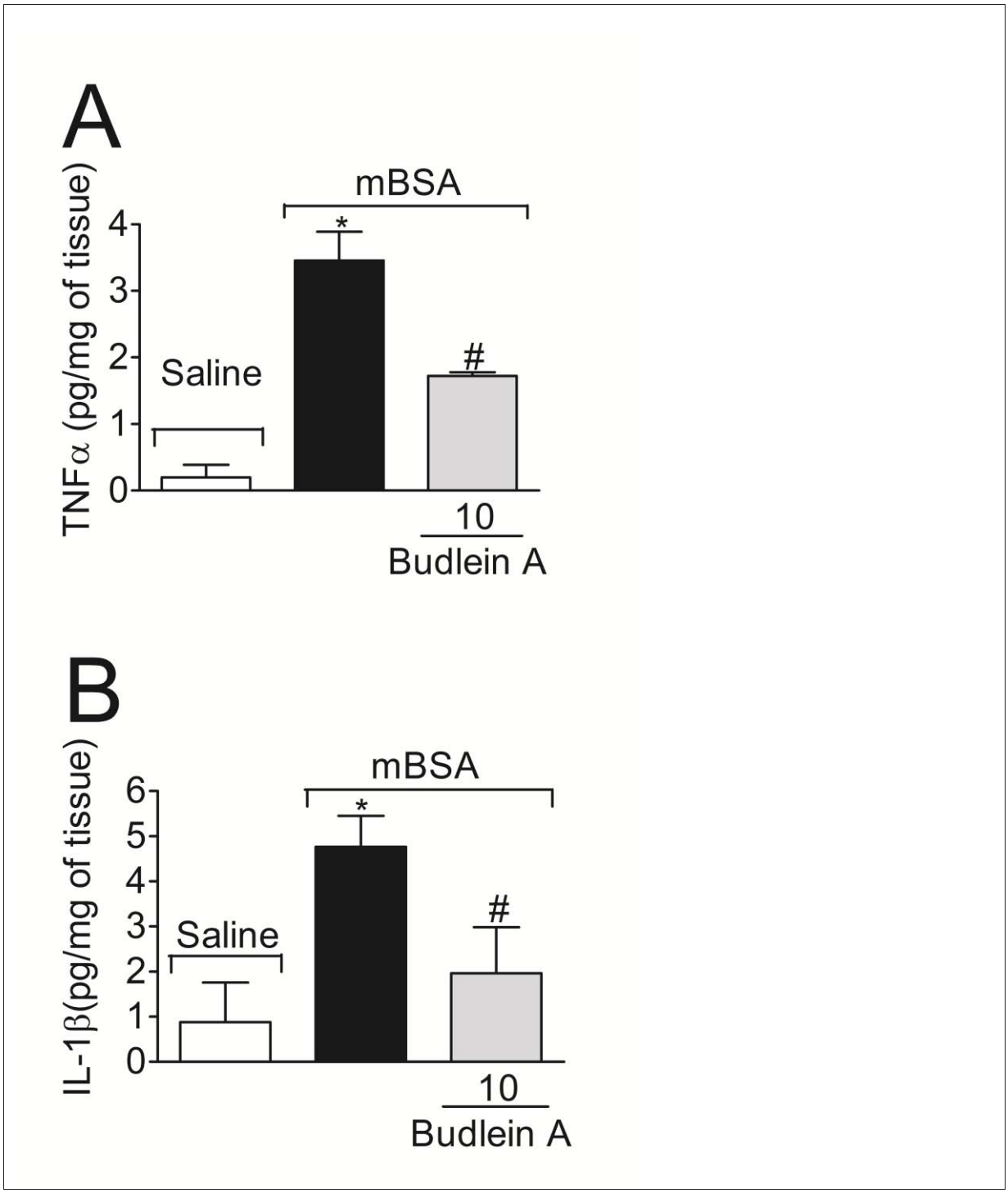

A
Neutrophils X 10^6/mg of tissue (MPO activitie)
mBSA
Sal
18
9
0
Im
Sl
2
1
10
Dexa
Budlein A
B
ALT (U/ml)
15
10
5
0
C
AST (U/ml)
80
60
40
20

Figure 4


A
TNFα (pg/mg of tissue)
mBSA
*
#
Saline
0
1
2
3
4
10
Budlein A
B
IL-1β(pg/mg of tissue)
mBSA
*
#
Saline
0
1
2
3
4
5
6
10
Budlein A

ANEXO 2:

Reduction of gouty arthritis-induced inflammation in mice by the sesquiterpene lactone budlein A: inhibition of cytokines production.

Reduction of gouty arthritis-induced inflammation in mice by the sesquiterpene lactone budlein A: inhibition of cytokines production.

[1]Zarpelon, AC; [2]Arakawa, NS; [3]Souto, FO; [4]Amaral, FA; [3]Ferreira, SH; [3]Cunha, FQ; [2]Da Costa, FB; [4]Teixeira, MM; and [1]Verri, WA, Jr.

[1]Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid PR 445, Km 380 Cx. Postal 6001, 86051-990, Londrina, Parana, Brazil. Fax: + 55 43 3371-4267, Tel: + 55 43 3371-4387. [2]Department of Pharmaceutical Sciences, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil. [3]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil. [4]Imunofarmacologia, Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Running title: Budlein A inhibits inflammatory pain.

***Author for correspondence:** Waldiceu A. Verri Jr (waldiceujr@yahoo.com.br or waverri@uel.br). Present address: Departamento de Patologia, Centro de Ciências Biologicas, Universidade Estadual de Londrina. Rod. Celso Garcia Cid Pr 445, KM 380, Cx. Postal 6001, 86051-990, Londrina, Parana, Brazil. Fax: + 55 43 3371-4387, Tel: + 55 43 3371-4979

Abstract

Background and purpose: Gout is an extremely painful and recurring form of inflammatory arthritis causing high morbidity and loss of life quality. The treatment is based on prevention of acute gouty arthritis by modulation of metabolism and anti-inflammatory treatment in acite events. Cytokines are clearly involved in gouty arthritis. In this sense, a drug that inhibits cytokine production would be a suitable strategy. We have previously demonstrated that budlein A, a sesquiterpene lactone inhibits cytokine production in carrageenin inflammation. Therefore, we addressed whether budlein A inhibits gouty arthritis-induced inflammation and its mechanism.

Experimental approach: The gouty arthritis was induced by intraarticular (i.a.) injection of MSU (monosodium urate) crystals diluted in borate buffer. Mice were treated with budlein A (1 or 10 mg/kg, p.o.) 30 min before intrarticular injection of MSU crystals. Articular hypernociception, odema and neutrophil migrations were evaluated 15, 24 and 48h after stimuli administration. Cytokine (TNFα and IL-1β) levels were determined by ELISA.

Key Results: The oral pretreatment with budlein A dose-dependently inhibited MSU-induced mechanical hypernociception, oedema, total leucocyte and neutrophil recruitment, and cytokines production.

Conclusions: These results suggested that budlein A treatment might be a suitable therapeutic approach in gouty arthritis.

Keywords: Budlein A, rheumatoid arthritis, pain, inflammation, hyperalgesia.

List of Abbreviations: Mono-sodium urate (MSU), Sesquiterpenes lactones (STLs), *Viguiera robusta (V. robusta)*, grams (g), intraarticular injection (i.a.),

Tumor necrosis factor alpha (TNFα), Interleukin-1 beta (IL-1β), Keratinocyte-derived chemokine (KC/CXCL1), Intraperitoneous (i.p.), Orally gavage (p.o.), Non steroidal anti-inflammatory (NSAIDs).

Introduction

Gout is a form of arthritis caused by a variety of known and unknown metabolic defects that ultimately result in accumulation of monosodium urate crystals (MSU). Acute gout is a common cause of arthritis, affecting approximately 1% of the adult population and triggered by specific events such as trauma, surgery, intercurrent illness, excess alcohol intake or drugs that alter serum urate levels (Sabina et al., 2010). MSU accumulates, in general, inside and around the joints causing inflammatory reactions that are clinically detected as intense pain, swelling and reddening of the skin (Terkeltaub et al., 2003; Chia et al., 2008b).

Monocytes/macrophages phagocyte MSU crystals and produce cytokines such as interleukin-1β (IL-1 β), tumor necrosis factor alpha (TNFα) and interleukin-8 (IL-8). This initial response by resident cells has a central role in the initiation and propagation of the inflammatory response (Di Giovine et al., 1991). Nevertheless, the primary pathologic hallmark of gout is the neutrophil influx into the joint (Liu-Bryan et al., 2005; Chen et al., 2006). These cells accumulate in both the joint fluid and the synovial membrane where they phagocyte MSU crystals. However, these crystals are toxic to neutrophils resulting in membrane lysis, generation of superoxide, prostaglandin E_2, leukotrienes and cytokine production (Terkeltaub et al., 2004; Liu-Bryan et al., 2005; Chen et al., 2006; Chia et al., 2008).

At the cellular level, a fundamental mechanism that promotes MSU-induced inflammation is innate immune engagement of the crystals by plasma membrane receptors, including Toll-like receptors (TLRs) 2 and 4, on

mononuclear phagocytes. Consequent phagocytosis, and events that seem to include phagolysosome destabilization related to MSU-induced membrane lysis, generation of reactive oxygen species and lowering of intracellular potassium levels, promote activation of the NLRP3 (Terkeltaub, R. et al., 2010; Bryan et al., 2005).

Non-steroidal anti-inflammatory drugs (NSAID's - indomethacin, naproxen) and glucocorticoids are frequently used as first line therapies for acute gout and these agents are generally effective, however, they also present serious side effects such as gastrointestinal ulcers and bleeding (cyclooxigenase-1 inhibitors), reduction of renal perfusion and increased thrombotic events (cyclooxigenase-2 inhibitors), and hormonal dysregulation, respectively (Sabina et al., 2010). Therefore, a drug that inhibits NFκB activation without the adverse side effects of glucocorticods would be a suitable strategy. In this sense, the sesquiterpene lactone obtained from Viguiera robusta, budlein A, seems a promising strategy. Budlein A has been previously isolated from Viguiera buddleiaeformis (De Vivar et al., 1976) and *Viguiera arenaria* (Siedle et al., 2004). However, despite its first isolation in the seventies, there are few reports on its activities. Budlein A inhibits TNFα-induced NFκB activation in Jurkat cells as shown by the eletrophoretic mobility shift assay (Siedle et al., 2004). *In vivo*, we have shown that it inhibits carrageenin-induced paw inflammation by inhibiting cytokine production (TNFα and IL-1β) (Valerio et al., 2007). Our data on inhibition of leukocyte recruitment and cytokine production (Valerio et al., 2007) was reinforced by others, which also demonstrated reduction of adhesion molecules by budlein A treatment (Nicolete et al., 2009).

In the present study, it was addressed whether budlein A treatment would be a promising therapeutic strategy in a model of gouty arthritis in mice. Budlein A treatment inhibited MSU arthritis-induced knee pain, edema, and leukocyte recruitment. This inhibition of inflammation was related to inhibition of cytokine production.

Materials and Methods

Animals

The experiments were performed on male Swiss weighing between 20 and 25g. Mice were housed in temperature-controlled rooms (22-25°C), with access to water and food *ad libitum*. All experiments were conducted in accordance with animals' care and handling procedures were accordance with the International Association for Study of Pain (IASP) and with the approval of the Ethics Committee of the State University of Londrina (UEL) (protocol no 05761).

Plant Material

The *Viguiera robusta* were samples collected in Batatais, state of São Paulo in April 2001 by FBC. In Biology Institute, Universidade de Uberlândia, Uberlândia, MG, Brazil were identified the material. Avoucher specimen (FBC#105) is deposited at the herbarium of the Department of Biology, University of São Paulo, Ribeirão Preto, São Paulo, Brazil under the code SPFR 07155 (Da Costa et al., 2001). The purity of budlein A was determined by chromatographic and spectrometric methods. TLC was carried out using several eluent systems and two spray reagents (1% vanillin–sulphuric acid or concentrated sulphuric acid). A high performance liquid chromatography (HPLC) run was made using methanol–water 55:45 or acetonitrile–water 65:35 as mobile phase, a reversed phase (ODS) analytical column, flowrate 1.0 or 1.3 mL/min, and UV detection at λmax 225 and 265 nm, as described elsewhere (Da Costa et al., 2001). Only one compound was detected in the chromatographic analyses. The 13C NMR spectrum of budlein A showed 20 carbon atoms corresponding to its structure.

By means of chromatographic and spectrometric methods,we estimated that the purity of budlein A is between 95–98%, therefore suitable for these biological assays.

Induction of Acute Gouty Arthritis

The uric acid crystals (MSU) were prepared by adding 5 mg/mL of uric acid on borate buffer (0,1 M, pH 8,5). The solution was filtered through a 0.45 µm filter. After 48h, the supernatant was transferred to another recipient e kept at 37^{o}C for seven days. The resultant solution was centrifuged (10.000 rpm, 10 min) and the supernatant despised. At this stage, all the crystals were adhered on the wall of the recipient. Subsequently, the MSU crystals were washed with absolute alcohol and acetone. The resulting MSU crystals were kept on ambient temperature to crystals obtainment (Chen et al., 2006).

Evaluation of articular hypernociception

The articular hypernociception was evaluated by an electronic version of von Frey method. Mice were placed in acrylic cages a wire grid floor, and the stimulations were performed only when the animals were quiet (and with the four paws on the grid floor). This method consists of an electronic pressure-meter, with a force transducer fitted with polypropylene tip (Insight instruments, Ribeirao Preto, SP, Brazil). To evaluate the articular pain, we used a large tip (4.15mm^2), to exclude the subcutaneous effect (Guerreiro et al., 2008). An increase perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the femur-tibial joint followed of the

hind paw withdrawal. A digital analgesymeter recorded the intensity of the force applied when the paw was withdrawn, expressed in grams (g). The test was performed in the times indicated on figures.

Paw oedema test

The volume of the mice joint was measure with a calipter (Mitutoyo) before (zero time) the intraarticular stimulus with mBSA, and after the administration, in the times indicated. The amount of paw swelling was determined for each mouse and the difference between the times indicated on figures and the zero time. The oedema value was represented in mm.

In vivo neutrophil migration

Mice received MSU crystals or vehicle (borate-buffered plus uric acid) injection directly into the femur-tibial articular cavity. At the time-points indicated on figures, the articular cavities were washed 3 times with 3.3μL of saline with 1mM EDTA (Verri et al, 2010). The total number of neutrophils was determined in Neubauer chamber using Turk's solution. Differential cell counts were determined by Roselfeld stained slices using a light microscope and results were expressed as the number of neutrophils per cavity.

Cytokines measurement

After 15h of the i.a. injection of the inflammatory stimuli (MSU), animals were terminally anesthetized and the articular joint were removed and homogenized in 500μL of buffer. TNFα and IL-1β concentration were determined by ELISA

(Ebioscience, 88-7324-76 and 88-7013-76 respectively). The results were expressed as pg/mg of tissue.

Experimental protocol

Firstly, mice were treated with three doses of MSU (30, 100 and 300μg/i.a.) and the total leucocytes and neutrophil migration was evaluated 15, 24 and 48h after the stimuli. The intensity of hypernociception and oedema was evaluated after 1, 3, 5, 7, 15, 24, 48, 72 and 96h. The effect of budlein A on MSU-induced neutrophil migration, hypernociception and oedema was determined 15 h after challenge (time chosen by previous experiments). The joints were removed after 15 h and homogenized in 500μL of saline, and TNFα and IL-1β concentration were determined by ELISA assay.

Statistical analyses

The results are representative of two or three independent experiments and are presented as the mean ± standard error mean (s.e.m.) ($n = 6$ per group in each individual experiment). One-way ANOVA followed by Bonferroni's *t*-test was performed to evaluate the differences between responses. Statistical differences were considered to be significant at $P < 0.05$.

Results

MSU induces dose-dependent neutrophil recruitment.

In the first series of experiments, it was established the optimal dose of MSU to induce neutrophil migration in the knee joint (i.a. administration). Mice received i.a. injection of three doses of MSU, and total leukocytes (Fig. 1A), neutrophils (Fig. 1B), and mononuclear cells (Fig. 1C) per cavity were determined at indicated time points. There was disgnificant recruitment of total leukocytes, neutrophils and mononuclear cell at 15 and 24 h. The recruitment at 48h was significant for 100 and 300 µg of MSU/joint, but was less prominent than at 15 and 24 h. The peak of recruitment was observed at 15h and the doses of 100 and 300 µg of MSU/joint induced significant recruitment of all cellular types without significant differences between these doses. Therefore, 15h and 100 µg of MSU/joint were chosen for next experiments on cellular recruitment.

MSU induces dose-dependent hypernociception and oedema.

Mice received MSU i.art. (30, 100 and 300µg/20µL), and the intensity of mechanical hypernociception (Fig. 2A) and oedema (Fig. 2B) were evaluated. All doses of MSU induced significant mechanical hypernociception and edema (Fig. 2). However, there was significant differences only between 30 and 300 µg of MSU doses at some time points, and without differences comparing 100 and 300 µg of MSU/joint. Therefore, the dose of 100 µg/joint was chosen taking into account the results of Fig. 1 and Fig. 2.

Budlein A inhibits gouty arthritis-induced mechanical hypernociception,

oedema and neutrophil migration in a dose-dependent manner.

It was addressed whether Budlein A inhibits mechanical hypernociception, oedema and cell migration induced by MSU (i.a. administration). Mice were treated with budlein A (1 and 10mg/Kg, 30 min) or vehicle (20% of Tween 80 plus saline to complete 150 µL, p.o.) before MSU stimuli (100 µg/i.a.). After 15 h intensity of mechanical hypernociception (Fig. 3A), edema (Fig. 3B), total leukocytes (Fig. 3C), neutrophils (Fig. 3D) and mononuclear cells (Fig. 3E) were determined. The budlein A dose of 10mg/kg/p.o. significantly inhibited MSU-induced mechanical hypernociception (Fig. 3A) while no effect was observed with 1 mg/kg of budlein A. Similar profile was observed in edema (Fig. 3B) except that the effect of 1 mg/kg of budlein A presented significant inhibition of edema. Both doses of budlein A inhibited the gouty-arthritis induced cell recruitment to the joint. The inhibition of leucocytes and neutrophil recruitment by the dose of 10mg/Kg was significantly greater than the 1mg/Kg of budlein A (Fig. 3C-3D) and there was no difference between doses of budlein A effect regarding mononuclear cells recruitment (Fig 3E).

Budlein A inhibits cytokine production (TNFα and IL-1β)

Mice were treated with budlein A (10mg/Kg, p.o., 30 min) or vehicle (20% of Tween 80 plus saline to complete 150 µL) before MSU (100µg/i.a.) stimulus. After 15 h of MSU administration, joint samples were collected in PBS buffer containing protease inhibitors (500µL) for cytokine level determination by ELISA. Budlein A significantly reduced MSU-induced TNFα and IL-1β production in the joint of mice (Fig 4A and 4B).

Discussion and Conclusions

Gout is a rheumatic disease characterized by an intense inflammation secondary to monosodium urate crystal (MSU) deposition in joints and surrounding tissues. There is marked influx of neutrophils, oedema and pain as a consequence of increased cytokine production (Champion et al., 1987). In the present study, the sesquiterpene lactone obtained from *Viguiera robusta*, budlein A inhibited MSU-induced gouty arthritis in mice. This effect was accompanied by inhibition of cytokine production.

The gouty inflammation is characterized by massive recruitment of neutrophils to which MSU crystals are cytotoxic, sinovitis, edema and pain. There is increased production of cytokines that have a crucial role in the development of the inflammatory response (Landis and Haskard, 2001; Torres et al., 2009). Cytokines, like TNFα and IL-1β consist of an important group of mediators produced in gouty that are responsible for recruitment of neutrophils (Faccioli et al., 1990) and pain (Ferreira et al., 1988) in other models of inflammation. In gouty arthritis, the genetic deficiency of IL-1R1 reduces both neutrophil recruitment and hypernociception (Torres et al., 2009). This IL-1-dependent mechanism in gouty is relevant in the peritoneal cavity, air pouch and joint (Liu-Bryan et al., 2005; Chen et al., 2006; Torres et al., 2009), and this consistency of data suggests it is an important therapeutic target. In fact, anakinra (a commercial IL-1 receptor antagonist [IL-1ra] that differs by 1 amino acid from the endogenous form of IL-1ra) diminishes gouty arthritis inflammation (So et al., 2007; McGonagle et al., 2007). IL-1β is produced upon inflammossome activation and up regulation by NFκB activation as well as

induces IL-1R1-dependent activation of NFκB. In agreement, inhibition of NFκB activation by glucocorticoids is also a therapy in acute gouty arthritis (Torres et al., 2009). Furthermore, it has been shown that budlein A inhibits TNFα-induced activation of NFκB using the electrophoretic mobility shift assay (Siedle et al.,)). Therefore, it is likely that inhibition of NFκB activation is an important mechanism in budlein A anti-inflammatory action. In fact, other sesquiterpene lactones also inhibit NFκB activation, and because of this activity they are in clinical trials for some cancer treatment (REFERENCIA REVIEW drug discovery).

In the present study, budlein A reduced the inflammation (e.g. neutrophil recruitment, edema and hypernociception) in a model of gouty arthritis. It was also detected that budlein A treatment reduced TNFα and IL-1β production in the knee joint of MSU-stimulated mice. These cytokines are important in the context of gouty arthritis as discussed above for IL-1β. In the case of TNFα, controversial results have been found, which might be related with a yet undetermined technical problem because the general concept is that inhibition of TNFα activity reduces gouty inflammation (So et al., 2007). Therefore, it is likely that budlein A is a promising strategy in gouty arthritis inflammation.

We have previously demonstrated that budlein A diminishes inflammatory overt pain-like behavior in models such as acetic acid-induced writhing response and formalin-induced flinches (Valerio et al., 2007). Furthermore, budlein A inhibits carrageenin-induced paw edema, neutrophil recruitment, and mechanical hypernociception by a mechanism related to reduction of TNFα and IL-1β production in the paw tissue (Valerio et al., 2007). These results were recently reinforced by data demonstrating that budlein A

reduces expression of adhesion molecules, neutrophil recruitment and rolling using intravital microscopy (Nicolete et al., 2009).

A possible explanation for the activity of budlein A in carrageenin model and in the gouty arthritis model is that, at least in part, similar receptors are activated in both models, and therefore, similar intracellular signaling pathways are important in both models. In the gouty arthritis model, it has been shown that toll-like receptors (TLR)-2 and TLR-4 deficiency reduce MSU-induced inflammation in the air pouch (Liu-Bryan et al., 2005). In the carrageenin-induced paw inflammation model, we have unpublished data demonstrating that TLR-2 and HeJ (deficient in TLR-4 signaling) mice present reduced inflammation, including paw edema, mechanical hypernociception and neutrophil recruitment (Zarpelon A.C. et al., unpublished data).

Concluding, it was demonstrated that budlein A treatment reduces edema, leukocyte recruitment and mechanical hypernociception in a model of gouty arthritis. The mechanism involves diminished cytokine production. Taking together the present results on *in vivo* activity and mechanism, with data indicating that prolonged treatment with budlein A does not alter liver function (Zarpelon A.C. et al., unpublished data) or induces gastric lesions (Valerio et al., 2007), it is likely that budlein A treatment is a conceivable approach to control gouty arthritis-induced inflammation and merits further pre-clinical and clinical investigation.

Acknowledgment and Funding

This work was supported by grants from Conselho Nacional de Pesquisa (CNPq, Brazil), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Fundação Araucária. Ana Carla Zarpelon received a MSc degree fellowship from CNPq – Brazil.

List of References

Ahmed M, Rahman MT, Alimuzzaman M, Shilpi JA (2001) Analgesic sesquiterpene dilactone from Mikania cordata. Fitoterapia 72:919-921.

Arakawa NS, Schorr K, Ambrósio SR, Merfort I, Da Costa FB (2008) Further sesquiterpene lactones from Viguiera robusta and the potential anti-inflammatory activity of a heliangolide: inhibition of human neutrophil elastase release. Z Naturforsch [C] 63(7-8):533-538.

Arkene, S.A.L., Simon, O., Shelly, J., Gardener, M. (2006). 6-Shogaol reduced chronic inflammatory response in calcium pyro phosphate dihydrate crystals. J. Exp. Med. 133, 100.

Baeuerle PA, Baltimore D (1996) NF-kappa B: ten years after. Cell 87:13-20.

Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141-179.

Barnes PJ (2006) How corticosteroids control inflammation: Quintiles Prize Lecture 2005 Br J Pharmacol 148:245-254.

Campion, E.W., Glynn, R.J., Delabry, L.O. (1987). Symptomatic hyperuricemia. Risks and consequences in the normative aging study. Am. J. Med. 82, 421–426.

Conte FD, Barja-Fidalgo C, Verri WA, Jr, Cunha FQ, Rae GA, Penido C and Henriques MD (2008) Endothelins modulate inflammatory reaction in zymosan-induced arthritis: participation of LTB4, TNF-α, and CXCL-1. J Leukoc Biol 84:652-660.

Cunha TM, Verri WA Jr, Valério DA, Guerrero AT, Nogueira LG, Vieira SM, Souza DG, Teixeira MM, Poole S, Ferreira SH, Cunha FQ (2008) Role of cytokines in mediating mechanical hypernociception in a model of delayed-type hypersensitivity in mice. Eur J Pain 12(8):1059-1068.

De Jongh RF, Vissers KC, Meert TF, Booij LH, De Deyne CS, Heylen RJ: The role of interleukin-6 in nociception and pain. *Anesth Analg* 2003, 96**:** 1096-103, table.

Di Giovine, F.S., Malawista, S.E., Thornton, E., Duff, G.W. (1991). Urate crystals stimulate production of tumour necrosis factor alpha from human blood monocytes and synovial cless: cytokine mRNA and protein kinetics, and cellular distribution. J. Clin. Invest. 87, 175–181.

De Vivar AR, Diaz CGE, Bratoeff EA, Jimenez L (1976) The germacranolides of Viguiera buddleiaeformis structures of budlein-A and –B. Phytochemistry 15:525-529.

Chen CH, Shi Y, Hearn A, Fitzgerald K, Golenbock D, Reed G, Akira S, Rock KL. (2006). MyD88-dependent IL-1 receptor signaling is essential for gouty

inflammation stimulated by monosodium urate crystals. The Journal of Clinical Investigation, 116 (8).

Chia EW, Pearce N, Berridge MV, Larsen L, Perry NB, Sansom CE, Godfrey CA, Hanton LR, Lu GL, Walton M, Denny WA, Webb VL, Copp BR, Harper JL (2008). Synthesis and anti-inflammatory structure–activity relationships of thiazine–quinoline–quinones: Inhibitors of the neutrophil respiratory burst in a model of acute gouty arthritis. Bioorg. Med. Chem. 16: 9432–9442.

Chia EW, Grainger R and Harper JL. (2008b). Colchicine suppresses neutrophil superoxide production in a murine model of gouty arthritis: a rationale for use of low-dose colchicines. British Journal of Pharmacology: 153 1288–1295.

Faccioli LH, Souza GE, Cunha FQ, *et al.* Recombinant interleukin-1 and tumor necrosis factor induce neutrophil migration “in vivo” by indirect mechanisms. *Agents Actions* 1990;**30**:344–9.

Feltenstein MW, Schuhly W, Warnick JE, Fischer NH, Sufka KJ (2004) Anti-inflammatory and anti-hyperalgesic effects of sesquiterpene lactones from Magnolia and Bear's foot. Pharmacol Biochem Behav 79:299-302.

Huacuja RL, Carranco A, Guzman SA, Guerrero C (1993) Inactivation of SH groups with sesquiterpene lactones: effects on nuclear decondensation pattern / motility induced by heparin in human spermatozoa. Adv Contracept Deliv Syst 9:97-106.

Landis, R.C., Haskard, D.O. (2001). Pathogenesis of crystal-induced inflammation. Curr. Rheumatol. Rep. 3, 36–41. Pan, M.H., Hsieh, M.C., Hsu, P.C., Ho, S.Y., Lai, C.S., Wu, H., 2008. 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. Mol. Nutr. Food Res. 52 (12), 1467–1477.

Lemos HP, Grespan R, Vieira SM, Cunha TM, Verri WA, Jr, Fernandes KS, Souto FO, McInnes IB, Ferreira SH, Liew FY, Cunha FQ (2009) Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN production. Proc Natl Acad Sci USA.

Liu-Bryan R, Scott P, Sydlaske A, Rose DM, Terkeltaub R. (2005) Innate Immunity Conferred by Toll-like Receptors 2 and 4 and Myeloid Differentiation Factor 88 Expression Is Pivotal to Monosodium Urate Monohydrate Crystal–Induced Inflammation. Arthritis & Rheumatism, 52: (9) pp 2936–2946.

Rüngeler P, Castro V, Mora G, Gören N, Vichnewski W, Pahl HL, Merfort I, Schmidt TJ (1999) Inhibition of Transcription Factor NF-kB by Sesquiterpene Lactones: a Proposed Molecular Mechanism of Action. Bioorg Med Chem 7:2343-2352.

Sabina EP, Rasool MK, Mathew L, EzilRani P, Indu H. (2010). 6-Shogaol inhibits monosodium urate crystal-induced inflammation – An in vivo and in vitro study. Food and Chemical Toxicology 48 (2010) 229–235.

Rasool M, Varalakshmi P. (2006) Suppressive effect of *Withania somnifera* root powder on experimental gouty arthritis: An *in vivo* and *in vitro* study. Chemico-Biological Interactions 164: 174–180.

Siedle B, Garcia-Pineres AJ, Murillo R, Schulte-Monting J, Castro V, Rungeler P, Klaas CA, Da Costa FB, Kisiel W, Merfort I (2004) Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappaB. J Med Chem 47:6042-6054.

Terkeltaub, R. (2004). Pathogenesis and treatment of crystal-induced inflammation. In: Koopman, W.J., Moreland, L.W. (Eds.), Arthritis and Allied Conditions. Williams and Wilkins, Philadelphia, Lippincott, pp. 2357–2372.

Terkeltaub R. (2010). Update on gout: new therapeutic strategies and options. Nat. Rev. Rheumatol. 6, 30–38.

Torres R, Macdonald L, Croll SD. (2009). Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis. Ann Rheum Dis., 68: 1602-1608.

Valerio DA, Cunha TM, Arakawa NS, Lemos HP, Da Costa FB, Parada CA, Ferreira SH, Cunha FQ and Verri WA, Jr. (2007) Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: inhibition of cytokine production-dependent mechanism. Eur J Pharmacol 562:155-163.

Verri WA Jr, Cunha TM, Parada CA, Poole S, Cunha FQ, Ferreira SH (2006). Hypernociceptive role of citokines and chemokines: targets for analgesic drug development: Pharmacol. Ther. 112 (1): 116-38.

Weyand, C.M. (2000). The role of T cells in RA. Arch. Immun. Ther. Exp. 48 (5), 429–435.

Legends to Figures

Figure 1: MSU induced dose-dependent neutrophil recruitment. Mice received the intraarticular administration of MSU (30, 100, 300µg/20µL) or vehicle (borate-buffered plus uric acid). Afterwards (15, 24 and 48h), animals were sacrificed and the articular cavities were washed 3 times with 3.3µL of saline with 1mM EDTA, and the total number of leucocytes was determined in a Neubauer chamber diluted in turk's solution 1:2 (used to lyse the erythrocytes) (Panel A). Differencial cells counts were determined by Roselfelt stained slices using a light microscope and results were expressed as the number of neutrophils or mononuclear cells per cavity (Panel B and C, respectively). **P*<0.05 compared with the negative control (indicated as zero), #*P*<0.05 compared with negative control and the MSU dose of 30µg/20µL, **P<0.05 compared with MSU dose of 30 and 100µg/20µL). One-way ANOVA followed by Bonferroni's test.

Figure 2: MSU induced dose-dependent hypernociception and oedema. The mice received MSU i.a. via (30, 100 and 300µg/20µL) and the intensity of mechanical hypernociception was evaluated using the von frey method after the stimuli, on the times 1, 3, 5, 7, 15, 24, 48, 72, 96h (Panel A). In the same times we evaluated the oedema (Panel B). **P*<0.05 compared with the negative control (indicated as zero), #*P*<0.05 compared with negative control and the MSU dose of 30µg/20µL, **P<0.05 compared with MSU dose of 30 and 100µg/20µL). One-way ANOVA followed by Bonferroni's test.

Figure 3: Budlein A inhibited gouty arthritis-induced mechanical hypernociception, oedema and neutrophil migration in a dose-dependent manner. The mice were treated with Budlein A (1 and 10mg/Kg, 30 min) or vehicle (20% of Tween 80 plus saline to complete 150 µL, p.o.) before MSU stimuli (100 µg/i.a.) and the intensity of mechanical hypernociception was evaluated 15 h after (Fig. 3A). In the same time, we evaluated the oedema (Fig. 3B). Afterwards the animals were sacrificed and the articular cavities were washed 3 times with 3.3µL of saline with 1mM EDTA. The total number of neutrophils was determined in a Neubauer chamber diluted in turk's solution 1:2 (used to lyse the erythrocytes) (Fig. 3C). Differencial cells counts were determined by Roselfeld stained slices using a light microscope and results were expressed as the number of neutrophils and mononuclear cells per cavity (Fig. 3D and E). *P<0.05 compared with the negative control, #P<0.05 compared with positive control (MSU dose of 100µg/20µL), **P<0.05 compared with Budlein dose of 1mg/Kg. One-way ANOVA followed by Bonferroni's test.

Figure 4: Budlein A inhibits cytokine production (TNFα and IL-1β) Mice were treated with Budlein A (10mg/Kg, p.o., 30 min) or vehicle (20% of Tween 80 plus saline to complete 150 µL) before MSU (100µg/i.a.) stimulus. After 15h of administration, the joint were collected in saline (500µL) for cytokine level determination by ELISA. *P<0.05 compared with the negative control, #P<0.05 compared with positive control (MSU dose of 100µg/20µL). One-way ANOVA followed by Bonferroni's test.

Figure 1

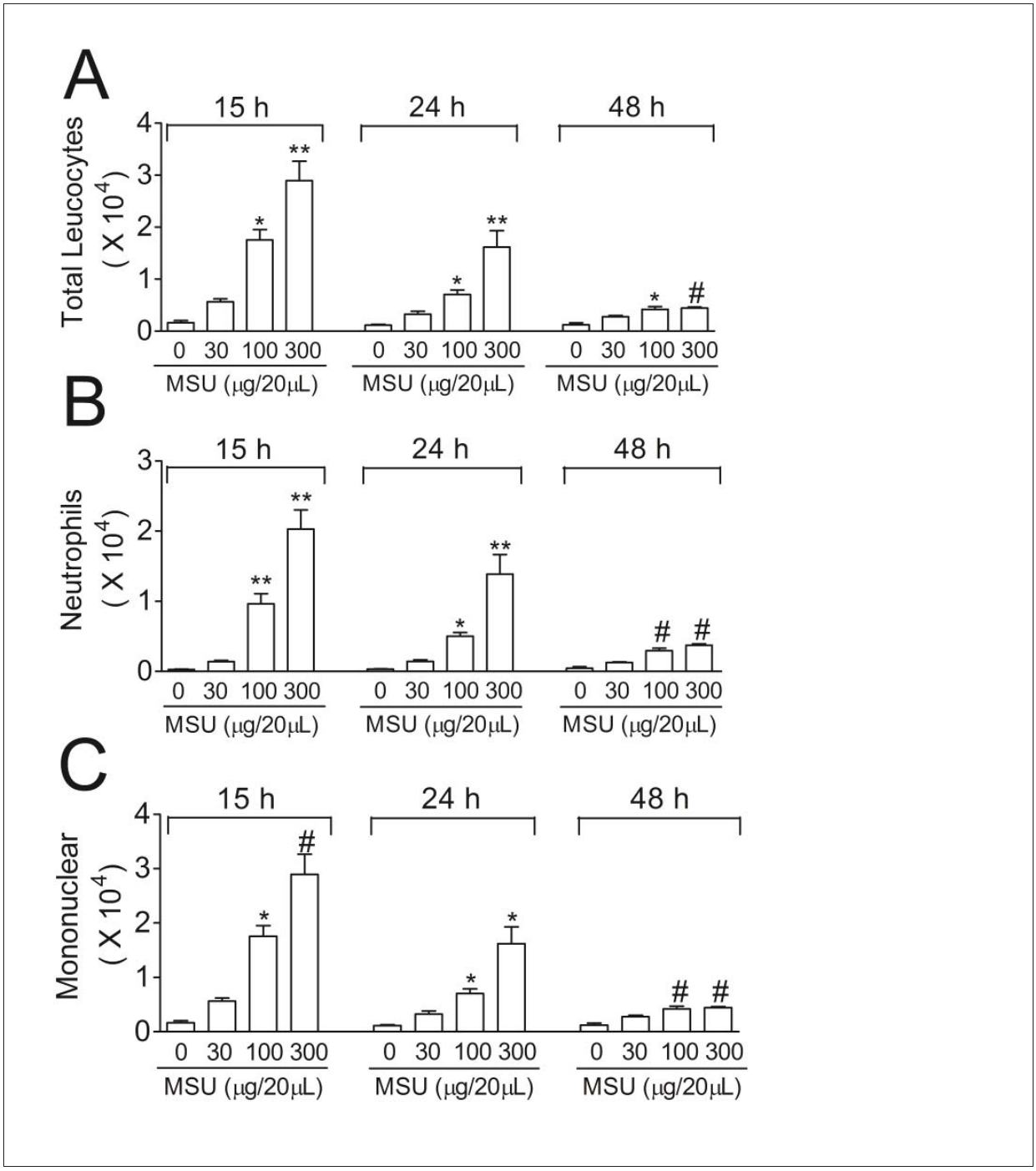

A
15 h
24 h
48 h
Total Leucocytes
(X 10^4)
0 1 2 3 4
0 30 100 300
MSU (μg/20μL)
B
Neutrophils
(X 10^4)
0 1 2 3
C
Mononuclear
(X 10^4)
0 1 2 3 4

Figure 2

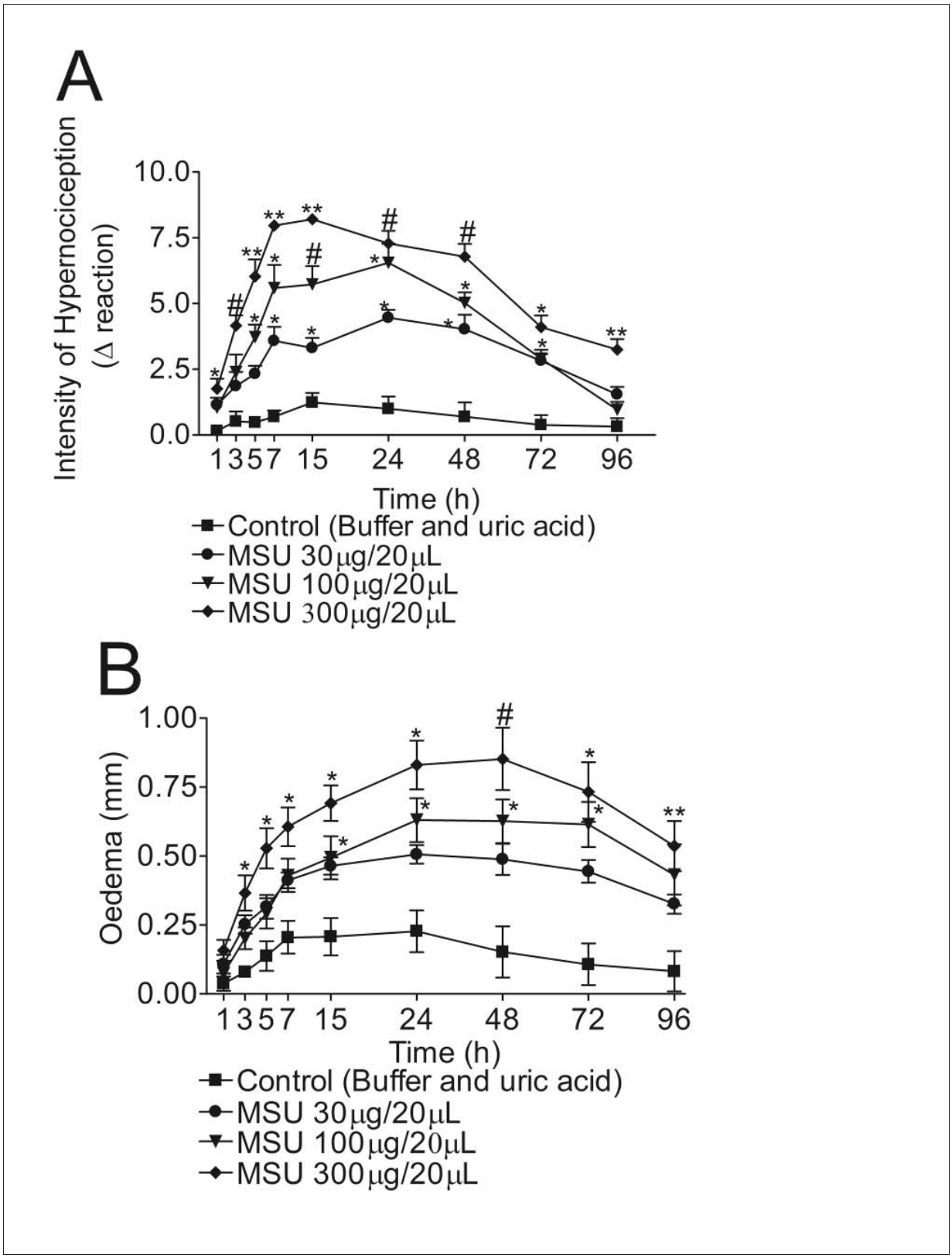

A
Intensity of Hypernociception
(Δ reaction)
10.0
7.5
5.0
2.5
0.0
1 3 5 7 15 24 48 72 96
Time (h)
Control (Buffer and uric acid)
MSU 30μg/20μL
MSU 100μg/20μL
MSU 300μg/20μL
B
Oedema (mm)
1.00
0.75
0.50
0.25
0.00
1 3 5 7 15 24 48 72 96
Time (h)
Control (Buffer and uric acid)
MSU 30μg/20μL
MSU 100μg/20μL
MSU 300μg/20μL

Figure 3

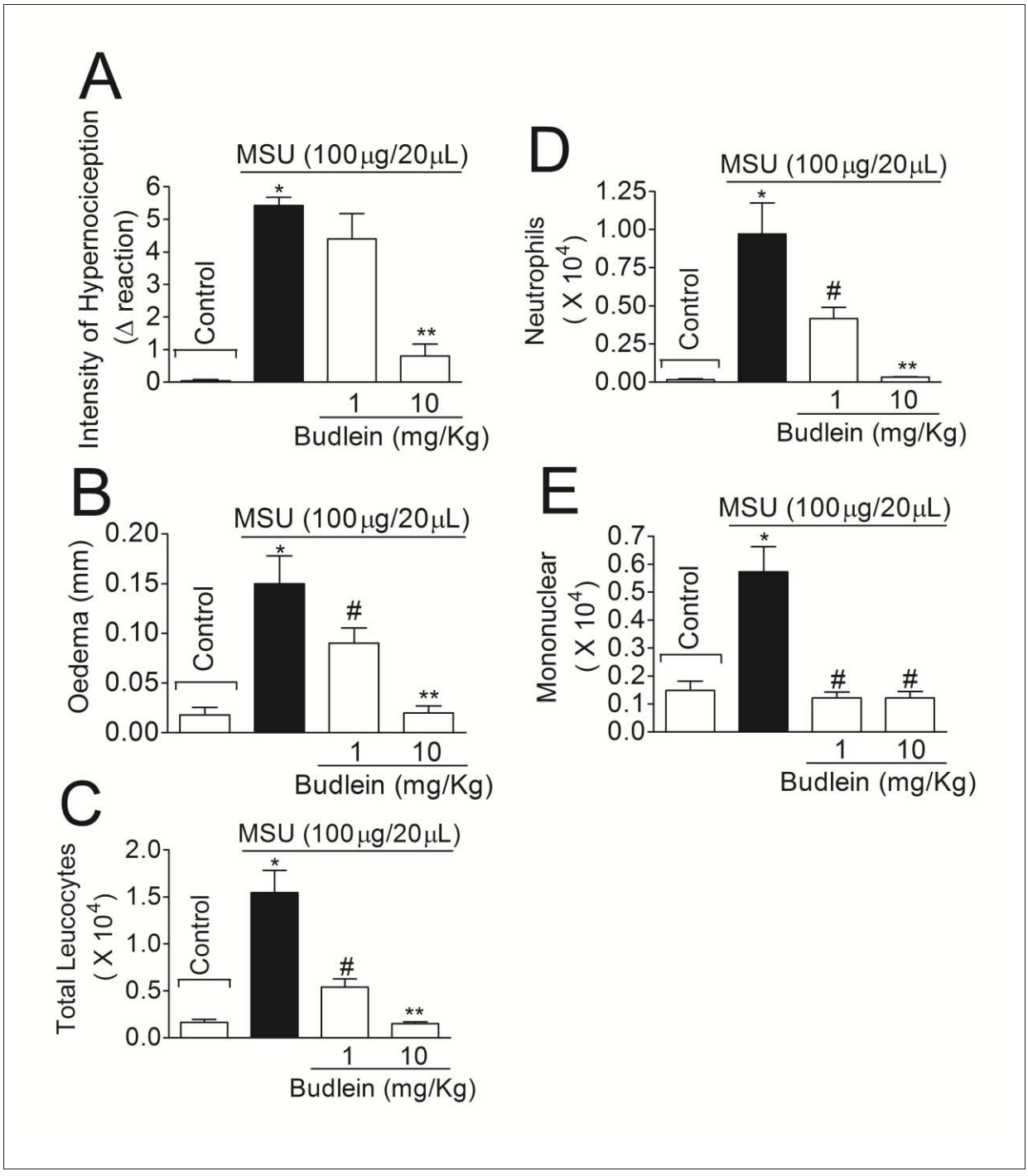
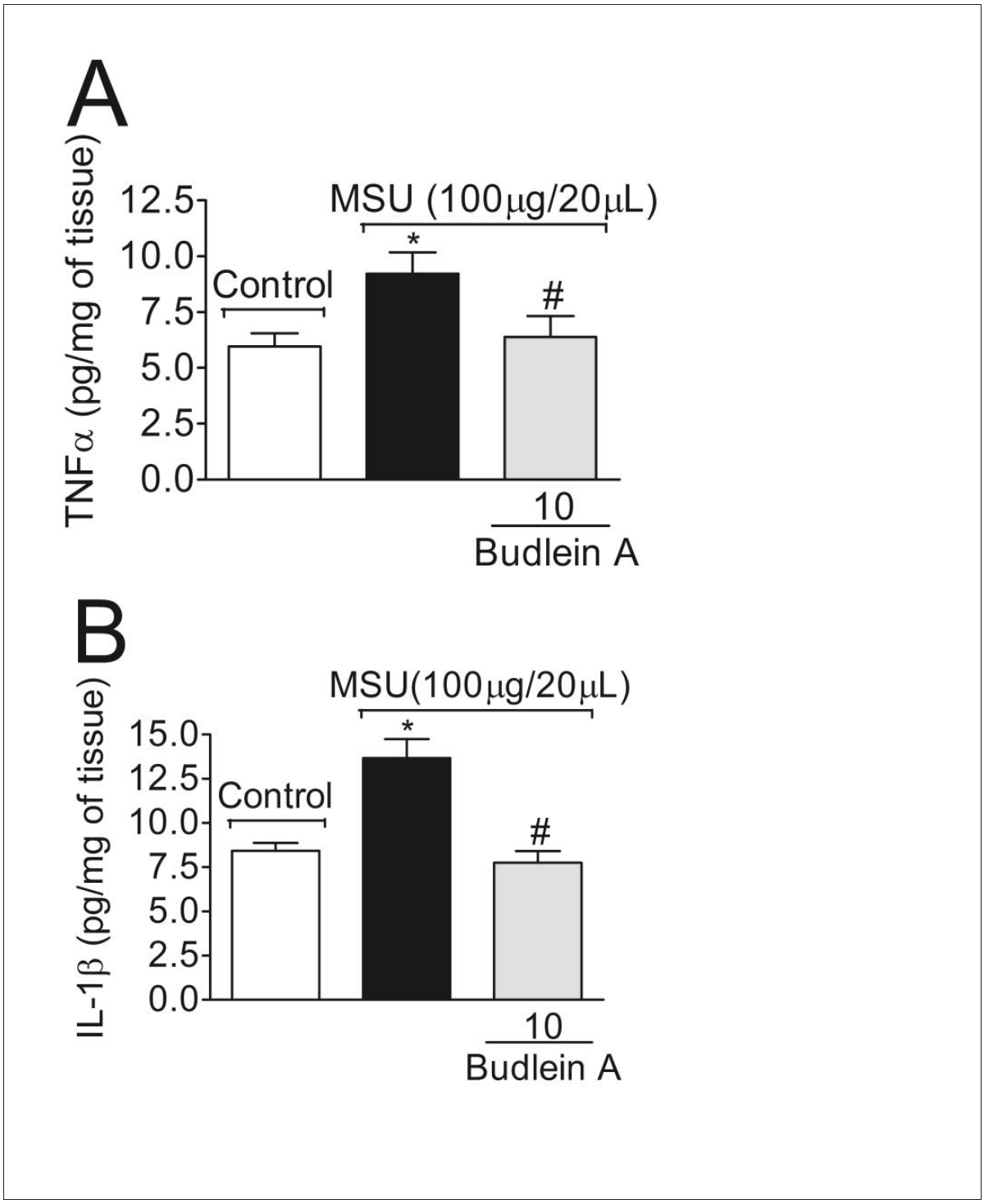

A
Intensity of Hypernociception
(Δ reaction)
MSU (100μg/20μL)
Control
Budlein (mg/Kg)
B
Oedema (mm)
C
Total Leucocytes
(X 10^4)
D
Neutrophils
(X 10^4)
E
Mononuclear
(X 10^4)

Figure 4


A
TNFα (pg/mg of tissue)
12.5
10.0
7.5
5.0
2.5
0.0
MSU (100μg/20μL)
*
#
Control
10
Budlein A
B
IL-1β (pg/mg of tissue)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
MSU(100μg/20μL)
*
#
Control
10
Budlein A

Conflicts of Interests

The authors declare that they have no competing interests.